Vol. 6. No. 2. JUNE 1951

UNIVERSITY
OF MICHIGAN
JUN 25 1951
MEDICAL
LIBRARY

BRITISH JOURNAL OF PHARMACOLOGY AND CHEMOTHERAPY

EDITED FOR
THE BRITISH PHARMACOLOGICAL SOCIETY
BY
J. H. BURN F. HAWKING
A. D. MACDONALD J. W. TREVAN E. J. WAYNE
J. H. GADDUM (Chairman) H. R. ING (Press Editor)
In co-operation with the EDITOR of the British Medical Journal

LONDON
BRITISH MEDICAL ASSOCIATION
TAVISTOCK SQUARE, W.C.1

YEARLY SUBSCRIPTION (4 NUMBERS) 30s. U.S.A. $5.00 SINGLE NUMBERS 7s. 6d.

NOTICE TO CONTRIBUTORS

The JOURNAL, which appears quarterly, publishes original work in all branches of pharmacology and experimental chemotherapy, including the biochemical and pathological aspects of the subject. Provision is also made for short communications and for brief notes on technique. Papers submitted for publication and all editorial communications should be addressed to Dr. H. R. Ing, Department of Pharmacology, Oxford.

Papers submitted to the JOURNAL are accepted on the understanding that they have not been and will not be published in whole or in part in any other journal; they are subject to editorial revision.

MSS. and illustrations should bear the name and full address of the author to whom proofs are to be sent. Papers should be in double-spaced typing on one side only of quarto or foolscap sheets, with a margin of not less than 1½ in. Papers must be as concise as possible, and only recognized abbreviations should be used.

In stating the object of the research the author should refer to previous work only if it has a direct bearing on the subject of the paper; as a rule a complete historical review is unnecessary. A paper should conclude with a brief but self-explanatory summary giving in numbered paragraphs the results and conclusions described in the paper; it should not exceed 1-20th of the length of the paper.

TABLES.—Tables are to be typed on separate numbered sheets and referred to in the text by Roman numerals. Tables should be comprehensible without reference to the text.

ILLUSTRATIONS.—Illustrations are to be referred to in the text by Arabic numerals. Legends or captions are to be typed on separate sheets and numbered correspondingly; they should render the illustrations comprehensible without reference to the text. Line drawings and graphs, which should be about twice their intended final size, are to be carefully drawn in Indian ink on tracing linen, or Bristol board, or stout smooth white paper; blue-lined graph paper of good quality may be used for graphs. Letterings of drawings and graphs must be lightly written in pencil. Lettering on kymograph records should be large enough to be legible on photographic reproduction. When half-tone reproduction of *x*-ray or other illustrations is required the author is advised to send the original film unless he wishes to bring out special points in a print of his own choice. Photographs and photomicrographs should be printed on glossy paper, should be larger than the size desired for reproduction, and, if transmitted through the post in a tube, should be rolled with the picture outside.

REFERENCES.—References should be arranged according to the Harvard system. In the text the year of publication must follow the author's name, more than one paper in any one year being indicated by a small letter (a, b, c) after the date. At the end of the paper references should be arranged in the alphabetical order of the authors' names and should include: author's name, followed by initials; year of publication, in parentheses; title of publication, underlined, and abbreviated in accordance with the *World List of Scientific Periodicals*; volume number in Arabic numerals underlined with a wavy line to indicate bold type; the number of the first page in Arabic numerals, thus: Barger, G., and Dale, H. H. (1910–11). *J. Physiol.*, **41**, 19.

The BRITISH JOURNAL OF PHARMACOLOGY AND CHEMOTHERAPY should be referred to in the list of references as *Brit. J. Pharmacol.* When reference is made to a book the author's name and the date should be followed by: title, underlined; edition, volume number (vol.), and page (p. or pp.); town of origin; and the publisher, thus: Clark, A. J. (1940). *Applied Pharmacology*, 7th ed., p. 378. London: Churchill.

For further information on the preparation of MSS. for publication authors are recommended to study the "Suggestions to Authors" published by the Editorial Board of the *Journal of Physiology* (1945), **104**, 221.

PROOFS.—Contributors will receive one proof in page, but it will be assumed that all but verbal corrections have been made in the original MS.; an allowance at the rate of ten shillings per sheet of sixteen pages is made for alterations in the proof (printer's errors excepted), and contributors will be charged for any excess.

REPRINTS.—Fifty reprints of an article will, if desired, be given to an author or divided among the authors. A limited number of additional reprints at cost price can be supplied if application is made when the proofs are returned, and an estimate of costs can be obtained from the Publishing Manager, British Medical Association.

COPYRIGHT.—Papers which have been published become the property of the JOURNAL and permission to republish must be obtained from the Editors.

Brit. J. Pharmacol. (1951), **6**, 169.

THE RENAL EFFECTS OF URETHANE AND COLCHICINE IN ADULT RATS

BY

S. E. DICKER

From the Department of Pharmacology, University College, London

(Received December 22, 1950)

A study of the mode of action of sodium cyanate on the kidneys of adult rats has shown that its diuretic effect is due mainly to a decrease in the rate of water reabsorption by the tubules (Dicker, 1950). As Dustin (1947) and Schütz (1949) had shown that sodium cyanate has a pronounced antimitotic activity, it was thought of interest to investigate whether other antimitotic substances like urethane or colchicine would have a similar diuretic effect and if so how they would affect the renal mechanism.

METHODS

Experimental animals.—Adult male albino rats (weight 250–300 g.) were used; they were fed on a commercially prepared diet, described in a previous paper (Dicker, 1949a).

Experimental procedure for the determination of inulin clearances.—The routine procedure for inulin clearance estimations conformed with that described previously (Dicker and Heller, 1945; Dicker, 1949b): rats were injected subcutaneously with 2 ml./100 g. body weight of a 5 per cent (w/v) solution of inulin in saline. The beginning of the "urine collection period" was started 50–60 min. after the injection of inulin (Friedman, Polley, and Friedman, 1947; Dicker, 1949b), whether water was administered or not. Immediately after the end of the "collection period," the animals were anaesthetized, and blood was obtained from the carotid and jugular vessels and mixed with heparin. The inulin used was that of T. Kerfoot and Co.

Administration of water.—When given lukewarm tap water by stomach tube, the animals received either *one* dose of 5 ml./100 g. (Heller and Smirk, 1932a; Dicker and Heller, 1945; Dicker, 1946) or *three* doses of 5 ml./100 g. at hourly intervals (Birnie, Jenkins, Eversole, and Gaunt, 1949; Dicker and Ginsburg, 1950). The volume of urine excreted after administration of one dose of 5 ml./100 g. body weight was expressed as a percentage of the amount of water administered. In rats which received three doses of the standard amount of water, the urine volume was expressed as a percentage of the water load, i.e., as a percentage of the total water given minus that excreted before injection of the drug (Birnie *et al.*, 1949).

Analytical methods.—Inulin in plasma and urine was determined by the method of Smith, Goldring, and Chasis (1938). Chloride, sodium, and potassium in plasma and in urine were estimated as described previously (Dicker, 1949a).

Drugs.—Urethane was injected subcutaneously in a dose of 50 mg./100 g. rat (5 per cent w/v in saline). Colchicine solutions were prepared as described by Fleischman (1939) and injected intramuscularly in a dose of 0.1 mg./100 g. body weight.

Methods of calculation.—The rates of tubular reabsorption of water and electrolytes were expressed in two different ways: (*a*) as the reabsorption of water or electrolytes by the renal tubules *as a whole*; (*b*) as the reabsorption of water and electrolytes by the more distal parts of the tubules. This later method of calculation is based on the assumption that about 80 per cent of the glomerular filtrate is reabsorbed iso-osmotically in the proximal segment of the mammalian nephron, regardless of the filtration rate. (Walker, Bott, Oliver, and McDowell, 1941; Wesson and Anslow, 1948; Wesson, Anslow, and Smith, 1948; Duggan and Pitts, 1950.)

The rate of reabsorption in the tubules *as a whole* was expressed as percentage of the glomerular filtration rate (Dicker, 1946 and 1948). Thus for water reabsorption (T_w):

$$T_w = \frac{C_{In} - U}{C_{In}} \times 100 \quad \ldots\ldots (1)$$

Where C_{In} = inulin clearance/min./100 g. = glomerular filtration rate (or GFR), U = urinary volume/min./100 g. body weight.

For the calculation of the tubular reabsorption of electrolytes the following formula was used (Dicker, 1948):

$$T = C_{In} \times E_p - U \times E_u$$

which when expressed as percentage of the amount of ion E filtered became:

$$T_E = \frac{T}{C_{In} \times E_p} \times 100 \quad \ldots\ldots (2)$$

Where E_p = concentration of the ion E per 100 ml. plasma, E_u = concentration of the ion E/100 ml. urine.

In order to calculate the absorption of either water or electrolytes by the *distal* portion of the tubules the amount of filtrate which had been reabsorbed by the proximal tubules had to be taken into account. The proximal reabsorption $Pr = \frac{C_{In} \times 80.}{100}$

Hence, the amount available for distal reabsorption (F) was:

(*a*) for water: $$F_w = C_{In} - \frac{C_{In} \times 80}{100}$$

(*b*) for an electrolyte: $$F_E = C_{In} \times E_p - \left[\frac{(C_{In} \times E_p) \times 80}{100}\right]$$

The reabsorption by the distal portion of the tubules was then:

(*a*) for water: $$Tw_D = \frac{F_w - U}{F_w} \times 100 \quad \ldots\ldots (3)$$

(*b*) for an electrolyte: $$Te_D = \frac{F_E - U \times E_u}{F_E} \times 100 \quad \ldots\ldots (4)$$

Statistical treatment.—Results are given as means and standard errors. Fisher's (1944) test for small samples was used for estimating the significance of means. The probability of P for t was obtained from the tables of Fisher and Yates (1943).

Results

Non-hydrated rats. I. Urethane

General effects of urethane on rats.—Rats injected with urethane showed signs of sleepiness and drowsiness, but no real anaesthesia: they reacted to prodding; their movements, however, were slow. No lachrymation was observed as with sodium cyanate (Birch and Schütz, 1946).

TABLE I

RENAL EFFECTS OF INJECTIONS OF URETHANE, COLCHICINE, AND SODIUM CYANATE IN HYDRATED AND NON-HYDRATED RATS

The figures are mean results with their standard errors. Number of animals in parentheses

		GFR (inulin clearance) ml./100 g./min.	Mean amounts of filtrate available for distal reabsorption. *F.* ml./100 g./min.	Mean urine flow ml./100 g./min.	Distal reabsorption as percentage of *F.*	
					of water %	of Cl %
Non-hydrated rats	Controls	0.53±0.043 (11)	0.11	0.0137±0.0024 (11)	87.3±1.89 (11)	97.3±0.19 (10)
	Urethane	0.64±0.087 (14)	0.13	0.0242±0.0059 (14)	77.7±4.90 (14)	94.9±0.77 (9)
	Colchicine ..	0.29±0.017 (11)	0.06	0.0526±0.0069 (11)	19.8±5.50 (11)	89.9±1.12 (11)
	Sodium cyanate*	0.31±0.034 (14)	0.06	0.0173±0.0030 (14)	72.8±0.76	75.9±1.99
Hydrated rats	Controls — 5 ml. water/100 g.	0.49±0.018 (14)	0.10	0.0342±0.0034 (14)	65.0±3.10 (14)	96.0±0.57 (14)
	Controls — 15 ml. water/100 g.	0.79±0.021 (15)	0.16	0.0811±0.0052 (15)	33.1±4.85 (15)	95.3±1.57 (9)
	Urethane — 5 ml. water/100 g.	0.75±0.050 (9)	0.15	0.0296±0.0050 (9)	81.4±2.91 (9)	94.5±0.60 (9)
	Urethane — 15 ml. water/100 g.	0.73±0.018 (9)	0.15	0.0736±0.0061 (9)	49.4±4.60 (9)	96.8±1.06 (9)
	Colchicine — 5 ml. water/100 g.	0.30±0.019 (11)	0.06	0.0606±0.0070 (11)	14.2±4.73 (11)	87.5±1.01 (11)
	Colchicine — 15 ml. water/100 g.	0.33±0.028 (9)	0.07	0.0424±0.0034 (9)	31.4±7.36 (9)	95.3±0.65 (9)

* Data about sodium cyanate have been taken from Dicker (1950).

Effects of urethane on urinary excretion.—Water and food were withheld from rats for at least six hours before they were injected subcutaneously with urethane. Fig. 1 shows the results of a dose of 50 mg. urethane per 100 g. on the urinary volume of 18 rats, compared with that of controls injected with comparable volumes of saline.

Urethane had a significant diuretic effect which started about 60 min. after the injection and lasted for about one hour. It had also a significant effect on the urinary excretion of Cl and Na: the concentration of both ions increased, and hence their excretion was enhanced (Table II). There was no significant effect on the urinary concentration of K.

Inulin clearance estimations.—The mean value for inulin clearances (=GFR) in rats injected with urethane amounted to 0.64±0.087 (S.E. of mean of 14 observations) ml./100 g./min. and was thus higher than that of controls (Table I; $t = 2.150$, $P = 0.05(23)$).

TABLE II

EFFECTS OF URETHANE AND COLCHICINE ON THE VOLUME OF URINE AND ITS IONIC COMPOSITION IN NON-HYDRATED RATS

Results are means and their standard error. Number of animals in parentheses.

	Urine volume ml./100 g./3 hr.	Cl mg./ 100 ml.	Cl mg./100 g./ 3 hr.	Na mg./ 100 ml.	Na mg./100 g./ 3 hr.	K mg./ 100 ml.	K mg./100 g./ 3 hr.
Control rats ..	0.78 ±0.24 (26)	77.2 ±4.31 (26)	0.66 ±0.10 (26)	36.0 ±2.01 (10)	0.28 ±0.05 (10)	100.7 ±13.40 (10)	0.78 ±0.26 (10)
Rats injected with urethane, 50 mg./100 g. s.c.	1.70 ±0.12 (18)	111.4 ±8.51 (18)	1.89 ±0.12 (18)	50.0 ±5.76 (9)	0.85 ±0.12	134.3 ±11.35 (14)	2.28 ±0.64 (14)
Rats injected with colchicine 0.1 mg./100 g. i.m.	1.14 ±0.074 (18)	92.0 ±7.00 (18)	1.04 ±0.10 (18)			105.7 ±14.15 (9)	1.20 ±0.13 (9)

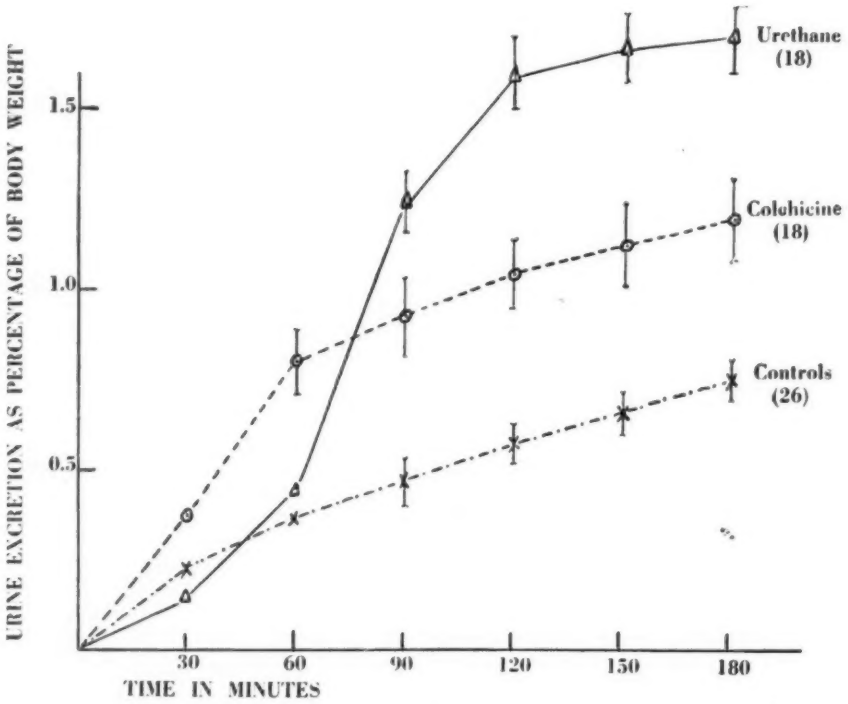


FIG. 1.—Effect of urethane and colchicine on the urine excretion of non-hydrated rats: Δ——Δ: rats injected with 1 ml./100 g. of a 5% solution of urethane; ⊙— — — — ⊙: rats injected with 0.1 ml./100 g. of colchicine (0.1% sol.); ×—·—·—·×: control rats injected with equivalent amounts of saline. The vertical lines indicate the standard errors. Number of animals in parentheses.

The tubular water reabsorption by the distal portion of the tubules was calculated on the assumption that 80 per cent of the filtrate was reabsorbed passively by its proximal portion (see equation 3): Tw_D amounted to $77.7 \pm 4.90(14)$; this value was not significantly different from that found in control animals (Table I; $t = 1.814$, $P > 0.05$).

In the same way, it could be shown that the mean value for the distal tubular reabsorption of chloride (TCl_D) was much the same in rats injected with urethane and in controls (Table I; $t = 0.425$, $P > 0.6$).

It can thus be concluded that the injection of urethane (50 mg./100 g. rat) had a marked diuretic effect in non-hydrated rats (Fig. 1) and that both the increased urinary volume and the enhanced chloride excretion were the result of an increased glomerular filtration rate, without any significant changes in the tubular reabsorptive activity for either water or chloride (Table I).

Non-hydrated rats. II. Colchicine

General effects of colchicine in rats.—Colchicine injected in a dose of 0.1 mg./100 g. rat had no immediate toxic effect. It had, however, a pronounced delayed toxic action: 12 rats out of 42 died eight to ten days after a single injection. Examination *post mortem* revealed haemorrhages in the small intestine of all these rats and large areas of congestion in the liver and the spleen; no macroscopical changes were seen in the kidneys.

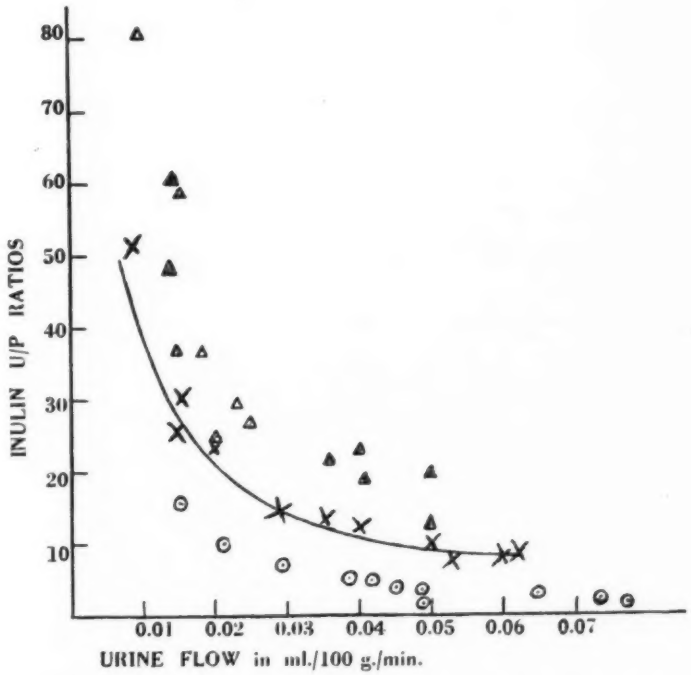


FIG. 2.—Effect of urethane and colchicine on the inulin U/P ratios, in non-hydrated rats. Δ: rats injected with urethane. ⊙: rats injected with colchicine. ×: control rats.

When, by mistake, the standard amount of water (5 ml./100 g.) was administered to the surviving animals about ten days after the injection of colchicine, about a third of them had an abnormally high urine output: 100 per cent of the administered water was excreted in less than 60 min.

Effects of colchicine on urinary excretion.—Water and food were withheld from rats for at least six hours before the rats were injected intramuscularly with colchicine. Fig. 1 shows the result of the injection of 0.1 mg. colchicine per 100 g. on the urinary excretion. There was a clear, though fleeting, diuretic effect: it started almost immediately after the injection and lasted for about 60 min. (Fig. 1).

Inulin clearance estimations.—The values for U/P ratios for inulin, and therefore the values for glomerular filtration rate, were lower than in controls (Fig. 2): the mean value for GFR in colchicine treated animals amounted to $0.29 \pm 0.017(11)$ ml./100 g./min. as compared with $0.53 \pm 0.043(11)$ ml./100 g./min. in controls. Concurrently the rate of water reabsorption was smaller than that of controls. Expressed as the rate of water reabsorption by the distal portion of the tubules, the mean value for Tw_D amounted to $19.8 \pm 5.50(11)$ per cent only (Table I). This result

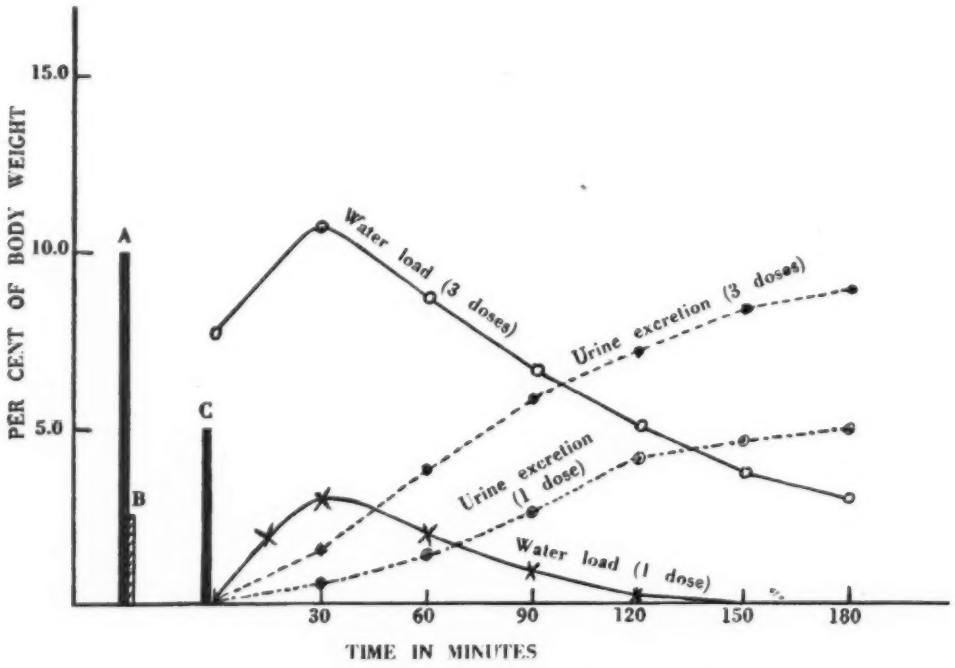


FIG. 3.—A comparison of the effects of the administration of one and three doses of 5 ml. water per 100 g. on the water load and urinary excretion of rats. A: amount of water given in 2 hours (= 10% body weight). B: amount of urine excreted in 2 hours (in % of the body weight). C. = volume of 5 ml./100 g. water: it represents either the third dose of water in rats receiving 15 ml./100 g. of water or the sole dose of water in rats receiving 5 ml./100 g. of water only. O——O: water load (% of body weight) of rats given 3 doses of water. × ——— ×: water load (% of body weight) of rats given 5 ml./100 g. of water. ●— — —● and ⊙— · — · —⊙ urine excretion (% of body weight) of rats hydrated with 3 and 1 doses of water respectively. Note the considerable increase of water load in rats which received 3 doses of water.

indicates that very little water was reabsorbed by the distal portion of the tubules. This was also suggested by the following facts observed at moderately high ranges of urine flow (e.g., beyond 0.05 ml./100 g./min.): (*a*) the values for *U/P* ratio for inulin did not change significantly (Fig. 2); (*b*) the mean value for the water reabsorption by the tubules as *a whole* ($=T_w$) amounted to 81.4±2.06(11) per cent, a figure which is very near to the theoretical value attributed to the iso-osmotic reabsorption by the proximal tubules; and (*c*) the calculated values for Tw_D were equal to zero (Fig. 4).

Effect of colchicine on excretion of chloride.—No significant increase of the chloride excretion could be noted in experiments where the urine collecting period extended up to 180 min. (Table II). Even during the short period when colchicine had its full diuretic effect (i.e., during periods when clearances were estimated) the chloride concentration of the urine of rats injected with colchicine remained comparable with that of controls, in spite of the fact that the mean filtration rate was decreased by about 40 per cent. This finding suggested that the chloride reabsorption by the tubules was decreased: Table I shows that values for TCl_D amounted to 89.9±1.12(11) per cent as compared with 97.3±0.19 per cent (t=4.378, P>0.001) in control animals.

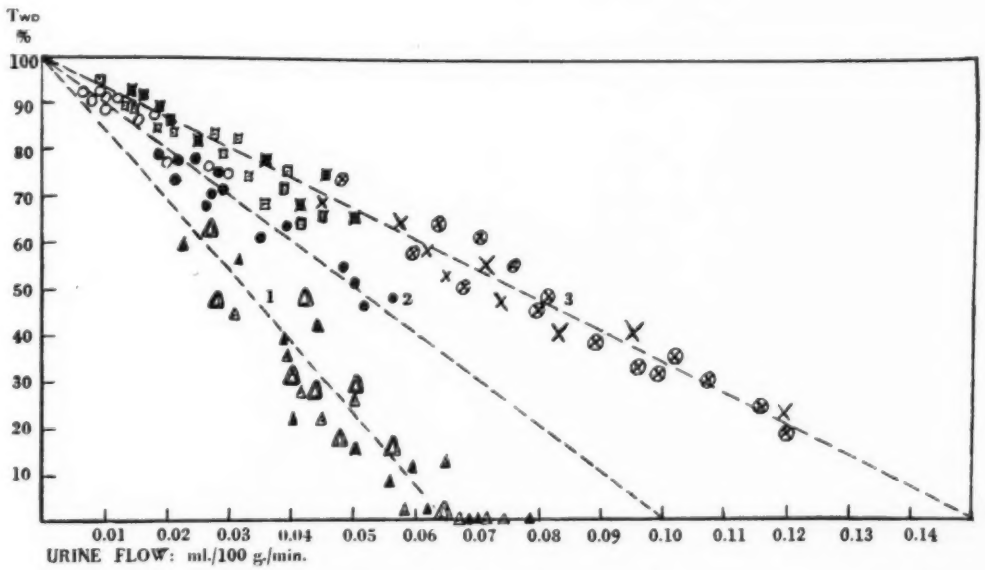


FIG. 4.—Relation between rates of water reabsorption by the distal portion of the tubules and rates of urine flow. The rates of water reabsorption are expressed as percentages of the amounts of filtrate available after 4/5 of it has been reabsorbed passively by the proximal portion of the tubules. 1, line representing mean results of water reabsorption for values of GFR = 0.3 ml./100 g./min.; 2, line representing mean results of water reabsorption for values of GFR = 0.5 ml./100 g./min.; 3, line representing mean results of water reabsorption for values of GFR = 0.75 ml./100 g./min. O: non-hydrated control rats, ● and ⊗ rats hydrated with 1 or 3 doses of 5 ml./100 g. of water, respectively. Δ: non-hydrated rats injected with colchicine, ▲ and ⏃ rats injected with colchicine and hydrated with 5 or 15 ml./100 g. of water, respectively. □: non-hydrated rats injected with urethane, ■ and × rats injected with urethane and hydrated with 5 and 15 ml./100 g. of water respectively.

Thus it would seem that during the diuretic effect of colchicine there was a very pronounced decrease of the reabsorptive capacity of the tubules for water, and to a lesser degree for chlorides.

Hydrated rats

Effect of an increased water load on the urine excretion

Urethane and colchicine had a clear diuretic effect in rats which had received no extra water (Fig. 1); they were, however, not dehydrated. The same observation had been made when investigating the action of sodium cyanate on the urine excretion of adult rats (Dicker, 1950). It was therefore of interest to see whether an increased water load would enhance the diuretic effect of these drugs. In order to increase the water load rats were given either *one* or *three* doses of 5 ml. tap water per 100 g. by stomach tube.

(*a*) *One dose of* 5 *ml. water per* 100 *g.*: The effects of this dose of water on the diuresis, water load, and renal functions have been described in detail in previous papers (Heller and Smirk, 1932a; Dicker and Heller, 1945; Dicker, 1946, etc.).

(*b*) *Three doses of* 5 *ml. water per* 100 *g.*: When expressed as a percentage of the actual water load, the urine excretion followed the usual pattern of a normal water diuresis. It will be seen, however, from Fig. 3 that the water load after administration of the third dose of water, i.e., at the beginning of the urine collection, was significantly higher than that observed after the administration of 5 ml. water per 100 g. only.

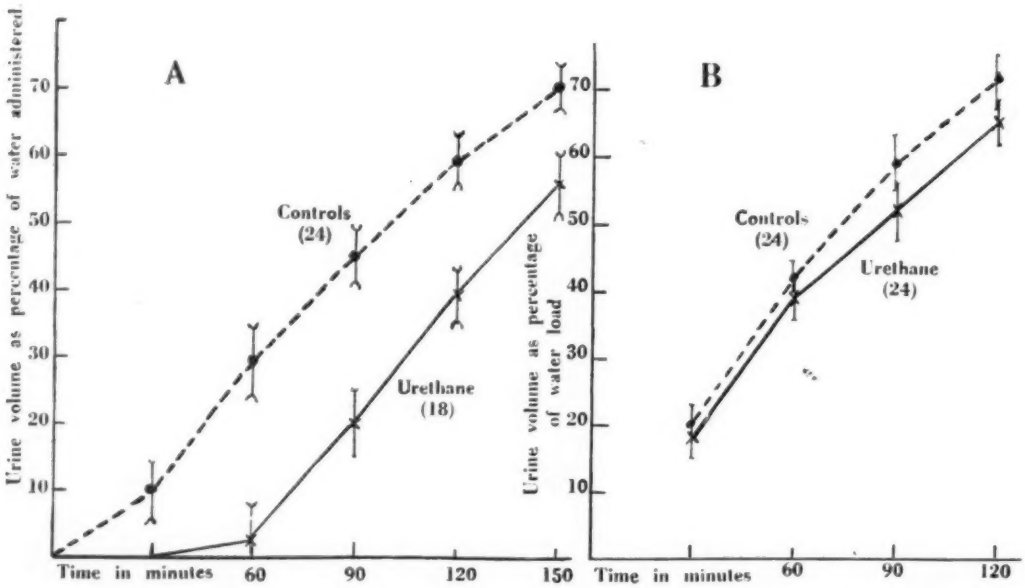


FIG. 5.—Effect of urethane on the urinary volume of rats which had received (A) 5 ml./100 g. of water, (B) 15 ml./100 g. of water. The urine volume is expressed in (A) as percentage of the amount of water administered; in (B) as a percentage of the water load. (For details see text.) The vertical lines indicate the standard errors.

The rate of glomerular filtration of rats which were given 15 per cent of their body weight of water was significantly raised. It amounted to $0.79 \pm 0.021(15)$ ml./100 g./min. Fig. 4 shows the values of water reabsorption by the distal portion of the tubules compared with those of rats which had received one dose of water only: it will be seen that at comparable rates of urine flow there was more water reabsorbed by the tubules of animals which were given 15 ml. water per 100 g. than by those which had received 5 ml. water per 100 g. only. This finding is consistent with that of an increased water load and explains why in the former series 75 per cent only of the initial water load was excreted after 180 min.

Effects of urethane on the renal functions of rats which received 5 *ml. water per* 100 *g.*

(1) *Water diuresis.*—The administration of urethane to hydrated rats (5 ml./100 g.) decreased their urine flow. It will be seen from Fig. 5A that there was a considerable delay in the onset of the water diuresis and that 50 per cent of the water administered was excreted after 140 min. only. This, however, could be explained by the fact

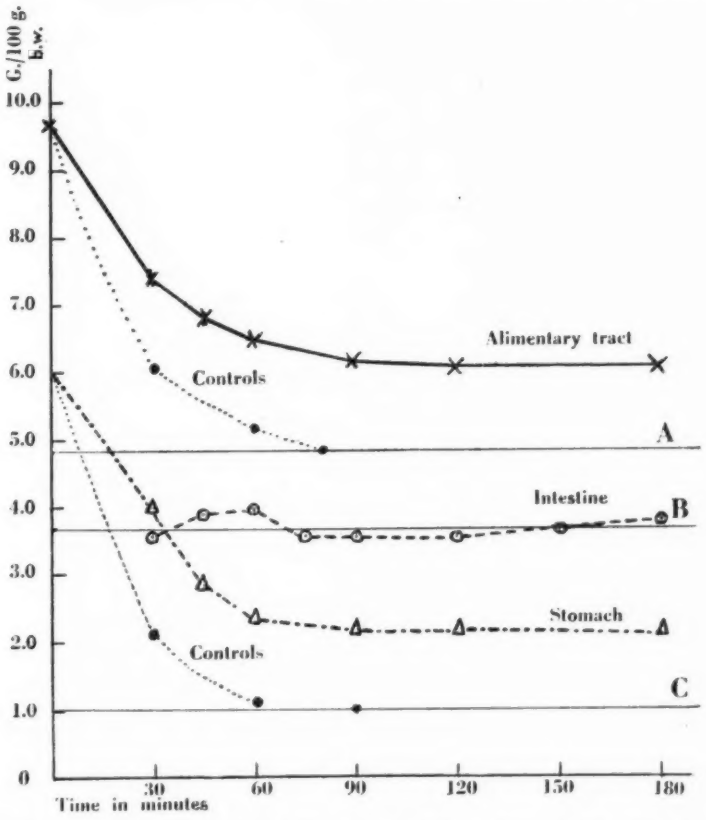


FIG. 6.—Average water absorption curves in rats injected with urethane. The average weight of the empty gastro-intestinal tract (A) was 4.7 g./100 g. body weight; 5 ml./100 g. of water was given, making 9.7 ml./100 g. the starting point of the absorption curve. The average weight of the empty intestine (B) was 3.7 g./100 g. rat and that of the empty stomach (C) was 1 g./100 g. rat. ×——× alimentary tract. ⊙— — —⊙: intestine. △—·—△: stomach. ●····●: water absorption in control rats.

that urethane retarded significantly the absorption of water from the stomach (Fig. 6). These results confirm those of Heller and Smirk (1932b).

(2) *Inulin clearances.*—The mean glomerular filtration rate of this series of animals amounted to $0.75 \pm 0.05(9)$ ml./100 g./min., which is not significantly different from that observed in non-hydrated rats injected with urethane (Table I).

It is thus clear that the administration of 5 ml. water per 100 g. did not increase the diuretic effect of urethane.

Effects of urethane on the renal functions of rats hydrated with three doses of water

In order to minimize any impairment of the absorption of water from the alimentary tract, urethane was injected one hour after the administration of the third dose of water. The mean water load at that time amounted to $8.9 \pm 0.49(36)$ ml./100 g. rat (Fig. 3). It will be seen (Fig. 5B) that the urine volume, expressed as a percentage of the water load, was much the same in rats injected with urethane as in controls. Likewise, no significant difference could be found between the rate of glomerular filtration and the rates of water and chloride reabsorption and the ionic concentration of urine ($\triangle$) in rats treated with urethane and in controls.

Effects of colchicine on the urine excretion of hydrated rats

(*a*) *In rats hydrated with* 5 *ml. water per* 100 *g.*—Colchicine was injected at the same time as water was administered. The total urine volume excreted in 180 min. was comparable with that of control animals. There was, however, a significant increase of the diuresis during the first hour following injection of the drug (Fig. 7A).

In contrast with urethane and sodium cyanate (Dicker, 1950), colchicine had no action on the absorption of water from the stomach and the gut.

Estimations of inulin clearances, of T_w and Tw_D showed that they were comparable with those found in non-hydrated rats: there was a marked decrease of the glomerular filtration accompanied by a pronounced decrease of values for T_w and Tw_D (Table I).

(*b*) *In rats hydrated with* 15 *ml. water per* 100 *g.*—(i) *Water diuresis*: Colchicine was injected 60 min. after the administration of the third dose of water, i.e., at a time when the water load was at its maximum (mean water load: $8.9 \pm 0.49(36)$ ml./100 g.). No significant difference could be noted in the rate of urine flow of rats whether injected with colchicine or not (Fig. 7B).

Chloride concentration as well as the freezing point ($\triangle$) were estimated in samples of urine collected at 1 hr. intervals in rats injected with colchicine and in controls: there was a significant decrease of the concentration of Cl in rats treated with colchicine ($t=2.202$) and the decrease of the freezing point, expressed in milli-osmol (C) indicated that the urine samples of colchicine treated animals were less concentrated than those of controls.

(ii) *Inulin clearances*: In spite of the administration of 15 ml. water per 100 g., the mean rate of urine flow and the mean values for GFR, T_w, Tw_D, T_{Cl}, and TCl_D were all comparable with those found in non-hydrated rats or in rats hydrated with one dose (5 ml./100 g.) of water only (Table I).

It is thus clear that colchicine acted quite independently of the degree of hydration of the animal and that in all animals the urine excretion was the result of a marked

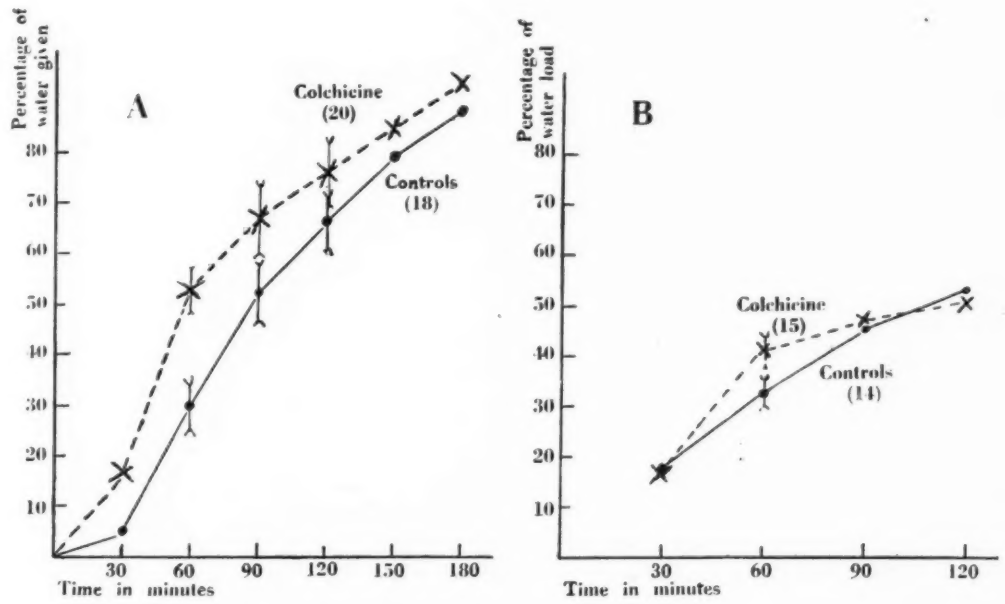


FIG. 7.—Effect of colchicine on the urinary excretion of hydrated rats. A: rats which received 5 ml./100 g. of water and were injected with colchicine. B: rats which received 3 doses of 5 ml./100 g. of water and were injected with colchicine 60 min. after the administration of the third dose of water. The urinary excretion is expressed in A as percentage of the amount of water administered; in B as percentage of the water load. ×——×: rats injected with colchicine. ●——●: controls. The vertical lines represent the standard errors. Number of animals in parentheses.

decrease of the tubular water reabsorption which counteracted the effect of a sharp fall in the glomerular filtration rate.

DISCUSSION

Urethane and colchicine both have an antimitotic activity, a property which they share with sodium cyanate (Dustin, 1947; Schütz, 1949) and to a certain extent with mercurial derivatives (Lettré, 1946; Meier and Schär, 1947). Though it is not known whether their antimitotic effect has any relation to their renal effects, it would appear that all four substances have a diuretic effect when injected into rats; however, mercurial derivatives have a diuretic effect in *hydrated* rats only (Dicker, 1946), while sodium cyanate increases the urine output when no extra water has been administered (Dicker, 1950). Urethane seems to act like sodium cyanate, while colchicine has a diuretic effect in non-hydrated rats and in rats which received one dose of water only (Fig. 7A). Further investigation has shown that colchicine produces a significant decrease of the filtration rate and that its diuretic effect seems to be due almost entirely to a decreased rate of water reabsorption by the tubules; this mode of action is reminiscent of that of sodium cyanate (Dicker, 1950). Urethane in non-hydrated rats on the other hand owes its diuretic effect both to an increase of the glomerular filtration rate and a decrease of the tubular water reabsorption; in this respect its action resembles that of mercurial derivatives (Dicker, 1946).

Is it permissible, or even correct, to assume that about 80 per cent of the glomerular filtrate is iso-osmotically reabsorbed by the proximal tubules, irrespective of the filtration rate? So far there has been only indirect evidence of such passive reabsorption of four-fifths of the glomerular filtrate (Walker *et al.*, 1941; Wesson *et al.*, 1948; Duggan and Pitts, 1950). However, even if this assumption were invalidated or had to be modified, the actual calculation used in this investigation (see equation 3), which consists in a uniform decrease of 80 per cent of the filtration rate, does not alter the conclusions materially. Presentation of the results in terms of this hypothesis has the advantage (or disadvantage) of magnifying changes in the rates of tubular reabsorption of water and chloride.

Changes in glomerular filtration rate raise an important issue, namely, the validity of inulin clearance estimations in animals injected with toxic drugs. Little criticism can be levelled against the finding of an *increase* of the filtration rate. In a *decrease* of the filtration rate, however, the decreased value of inulin clearance may be the result of a back diffusion of that substance, and if so inulin clearances would no longer represent a measure of filtration. No experiments were specifically carried out to investigate this question, but certain findings suggest that the decrease of inulin clearance found in rats injected with colchicine (or sodium cyanate) represents a real decrease of the filtration rate. Firstly, in rats injected with sodium cyanate, it has been shown that the filtration fraction was significantly lower than that of control animals, indicating a dilatation of the glomerular vessels, which would lead to a decrease of the glomerular filtration rate (Dicker, 1950). Secondly, in rats injected with colchicine, it was found that for the *same* volume of urine flow, the urine was regularly less concentrated (in terms of osmotic pressure). This could be due either to an intoxication of the tubular cells resulting in a back diffusion of solutes and of inulin, or to a decreased rate of water reabsorption. The hypothesis of a severe tubular intoxication leading to back diffusion of the solutes is ruled out by the finding that the urinary concentration of Cl and K remained unaffected (Table II) and that values for TCl_D were very much the same as those found in control rats (Table I). It would therefore seem more probable that colchicine acts specifically on the water reabsorption. At least one other drug, vasopressin, is known to have such a specific effect; it increases the rate of water reabsorption but has no effect on the chloride excretion (Dicker and Heller, 1946; Heller and Stephenson, 1950). If it be assumed, therefore, that colchicine affects the water reabsorption only, it is difficult to believe that the decreased value for the inulin clearance was due to a back diffusion of inulin through the tubular cells.

Summary

The effects of two antimitotic drugs (urethane and colchicine) on urine excretion were investigated (*a*) in non-hydrated adult rats, (*b*) in adult rats which had been given either 5 ml./100 g. or 15 ml./100 g. of water, the latter at hourly intervals.

A. Non-hydrated rats

1. The subcutaneous injection of urethane (1 ml./100 g. of a 5 per cent (w/v) solution of urethane) produced a significant increase of the urine excretion accompanied by an enhanced excretion of Cl and Na. The increased volume of urine was mainly the result of an increased glomerular filtration rate.

2. The intramuscular injection of colchicine (0.1 mg./100 g. rat) produced an increased urinary excretion, which started soon after the injection and ended in about an hour. There was no significant effect on the urinary excretion of Cl or Na. The enhanced diuresis was the result of a pronounced decrease of the tubular water reabsorption, the glomerular filtration (=inulin clearances) being markedly depressed.

B. Hydrated rats

1. The administration of 5 ml./100 g. of water had no effect on the glomerular filtration rate which remained comparable to that of non-hydrated rats. However, the administration of three doses of the standard amount of water produced a marked increase of the glomerular filtration rate.

2. The injection of urethane into hydrated rats (with one or three doses of water) resulted in a decreased water diuresis, due mainly to a decreased absorption of water from the alimentary tract.

3. The injection of colchicine did not impair the water absorption from the stomach and intestine. It produced a fleeting increase of the water diuresis. It had the same depressing effect on the glomerular filtration as in non-hydrated rats.

This investigation was started while working in the Department of Pharmacology, University of Bristol. I should like to express my sincere thanks to Professor H. Heller for granting me all facilities in his Department.

REFERENCES

Birch, K. M., and Schütz, F. (1946). *Brit. J. Pharmacol.*, **1**, 186.
Birnie, J. H., Jenkins, R., Eversole, W. J., and Gaunt, R. (1949). *Proc. Soc. exp .Biol., N.Y.*, **70**, 83.
Dicker, S. E. (1946). *Brit. J. Pharmacol.*, **1**, 194.
Dicker, S. E. (1948). *J. Physiol.*, **107**, 8.
Dicker, S. E. (1949a). *Biochem. J.*, **44**, 274.
Dicker, S. E. (1949b). *J. Physiol.*, **108**, 197.
Dicker, S. E. (1950). *Brit. J. Pharmacol.*, **5**, 13.
Dicker, S. E., and Ginsburg, M. (1950). *Brit. J. Pharmacol.*, **5**, 497.
Dicker, S. E., and Heller, H. (1945). *J. Physiol.*, **103**, 449.
Dicker, S. E., and Heller, H. (1946). *J. Physiol.*, **104**, 353.
Duggan, J. J., and Pitts, R. F. (1950). *J. clin. Invest.*, **29**, 315.
Dustin, P. (1947). *Nature, Lond.*, **159**, 794.
Fisher, R. A. (1944). *Statistical Methods for Research Workers.* 9th ed. London: Oliver and Boyd.
Fisher, R. A., and Yates, F. (1943). *Statistical Tables for Biological, Agricultural, and Medical Research.* 2nd ed. London: Oliver and Boyd.
Fleischman, W. (1939). *Endocrinology*, **25**, 798.
Friedman, S. M., Polley, J. R., and Friedman, C. L. (1947). *Amer. J. Physiol.*, **150**, 340.
Heller, H., and Smirk, F. H. (1932a). *J. Physiol.*, **76**, 1.
Heller, H., and Smirk, F. H. (1932b). *J. Physiol.*, **76**, 283.
Heller, H., and Stephenson, R. P. (1950). *Nature, Lond.*, **165**, 189.
Lettré, H. (1946). *Naturwissenschaften*, **3**, 75.
Meier, R., and Schär, B. (1947). *Experientia*, **3**, 1.
Schütz, F. (1949). *Experientia*, **4**, 133.
Smith, H. W., Goldring, W., and Chasis, H. (1938). *J. clin. Invest.*, **17**, 263.
Walker, A. M., Bott, P. A., Oliver, J., and McDowell, M. C. (1941). *Amer. J. Physiol.*, **134**, 580.
Wesson, L. G., and Anslow, W. P. (1948). *Amer. J. Physiol.*, **153**, 465.
Wesson, L. G., Anslow, W. P., and Smith, H. W. (1948). *Bull. Acad. Med., New York*, **24**, 586.

Brit. J. Pharmacol. (1951), **6**, 182.

AN IMPROVED APPARATUS FOR THE STUDY OF THE MOTILITY OF ISOLATED ORGANS

BY

CAMILLO BIANCHI AND GIOVANNI CERIOTTI
with the technical assistance of
MARIO ORTELLI

From the "Carlo Erba" Institute for Therapeutic Research, Biological Division, Milan

(Received December 7, 1950)

The extensive use of isolated organs in pharmacological research and in biological determinations of certain substances has led to an effort to improve upon the existing apparatus. During the washing of suspended organs chemical and mechanical disturbances occur which may alter their reactivity, especially when the organ fragment is washed by repeated filling and emptying of the suspension bath, so exposing the tissue to the air for considerable periods of time. We have devised a new type of suspension bath for isolated organs which overcomes this difficulty. It is based on the principle used by Zamboni (1940), Schild (1946, 1947), and by Fastier and Reid (1949) that the organ is washed by a constant stream of fluid entering from the bottom and flowing out from the top of the bath.

The apparatus, as shown in Fig. 1, consists of a support (A) to which cylindrical metal chambers (B) of different sizes can be screwed.* These are equipped with a discharge tube (C) and a level tube (D). The bottom of the support opens into a T-tube. The other arms of the T-tube are for the oxygen (E) and for the inflow (F) of the perfusion fluid. The latter is connected to a two-way stopcock with a long handle, through which the arm may be connected either to the elevated reservoir of perfusion liquid (H) or to the collecting flask (I).

The organ bath is made entirely of bronze with the inner surface gold-plated. Satisfactory results have also been obtained with a similar bath plated with pure tin. The graduated glass level tube (D) has an internal diameter of 5 mm. to avoid the capillarity phenomenon and is connected to the metal part with rubber tubing. When the bath is connected through the two-way stopcock to the reservoir, the perfusion fluid, at the desired temperature, flows in from the bottom and flows out at the top through the discharge tube, thus washing the suspended fragment without exposure to air. A fixed volume of fluid may be used by keeping the washing time constant. The volume of liquid in the bath may be regulated at will by turning the stopcock through 180° so that the fluid flows into the collector. The outflow may be stopped when the desired volume is attained as indicated on the level tube.

*Height 110 mm. Diameter 18 mm. Usable capacity about 20 c.c.
,, ,, ,, ,, 25 mm. ,, ,, ,, 50 c.c.
,, ,, ,, ,, 36 mm. ,, ,, ,, 100 c.c.

The suspension bath is immersed by means of a metal rod attached to it in a large thermostatic bath (70×50×50 cm.) at 37° C. A constant temperature is maintained by a " Vertex " thermostatic apparatus. The bath contains a copper warming coil for the perfusion fluid, gold-plated on the inside and of about one litre capacity (5.5 m. in length). The organ is placed on a movable hook (Fig. 1), which consists of a metal rod attached by a spring to the wall of the bath. This simple tool greatly facilitates the insertion and removal of the organ.

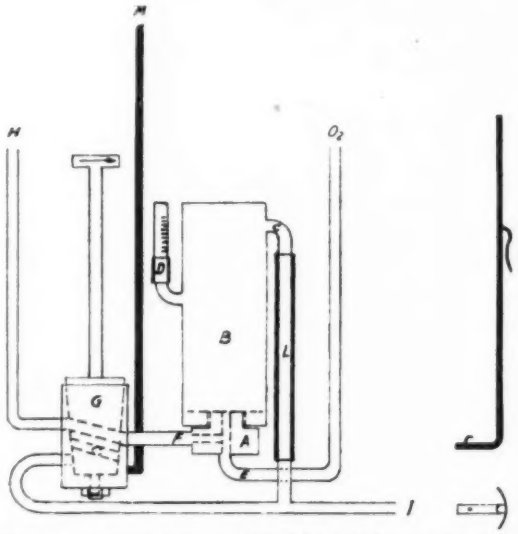


FIG. 1.—Organ bath and (right) hook for suspension of organ; for explanation see text.

In addition to the suspension bath, an attempt has also been made to improve the writing pen, and a pen has been devised which gives exact proportional enlargement of the movements of the organ and records in straight lateral writing.

The pens commonly used, both the lateral and the frontal writing types, are so constructed that the points where force and resistance are applied move in an arc of a circle. With the pen which we have devised the points remain constantly on the tangent to these arcs. This principle has been described by Zamboni and Vendramini (1947), who applied it only to the side of the lever where the acting force, i.e., the contracting organ, is exerted. By applying it also to the other side where there is resistance, i.e., to the writing point, the enlarged recording is exactly proportional to the movements of the organ. Lateral writing with very little friction is also achieved by this means and large displacements may be registered. The pen (Fig. 2) consists of a flat thin metal rod (A) moving on a fulcrum (B). The short arm is one-fifth the length of the long arm. To the end-points of both the arms, two thin metal arcs (C and D) are sealed whose radii correspond to the distances between the fulcrum and the end-points. Silk threads are attached to the upper points of the arcs and run through shallow grooves cut on the surface of the arcs. One (E) bears a metal hook to which the organ is attached; the other (F) is

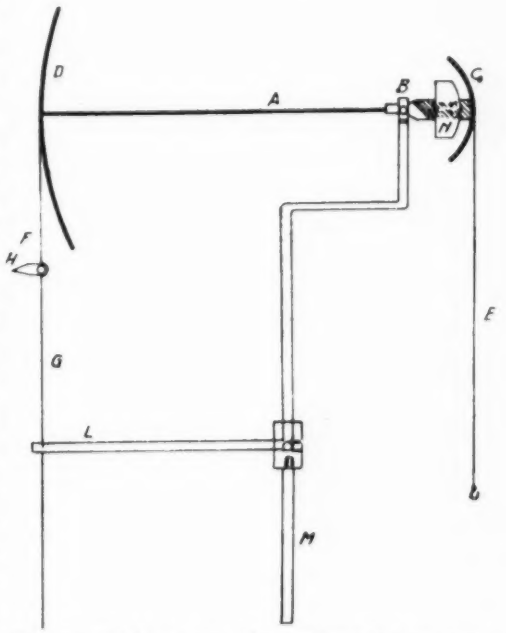


FIG. 2.—Writing pen; for explanation see text.

attached to a thin straight steel wire (G) through a small ring. A stylus (H) made of paper, cellophane, or celluloid is attached to the ring with sealing-wax. Weights may also be suspended from the ring, if necessary, to extend the organ. The metal wire runs through a hole in a rod (L) placed perpendicularly to it and attached to the support (M); in this way the stylus can only move in a vertical direction.

An accurate equilibration of the lever may be obtained by a weight (N) moving on a screw along the arm to which the organ is attached. The point of the stylus can be maintained in constant contact with the kymograph by simple torsion of the silk thread.

The pen has been used to register the contractions of isolated uterus and intestine of the rabbit, guinea-pig, and rat. There is a noticeable inertia, because of its weight, which makes it inadvisable to use the pen when frequent movements have to be recorded.

The apparatus described above has been in use for more than two years with satisfactory results.

Summary

A description of a complete apparatus for the study of the motility of isolated organs is reported. A suspension bath is described which permits the washing of the organ without exposure to the air.

A writing pen is also described which records exact proportional enlargement of the movements of the organ in straight lateral writing.

We wish to express our sincere thanks to Professor Luigi Butturini for his valuable suggestion during the elaboration of this apparatus.

References

Fastier, F. N., and Reid, C. S. W. (1949). *Brit. J. Pharmacol.*, **4**, 109.
Schild, H. O. (1946). *Brit. J. Pharmacol.*, **1**, 135.
Schild, H. O. (1947). *Brit. J. Pharmacol.*, **2**, 189.
Zamboni (1940). Atti Primo Congresso Soc. Ital. Farmacol.
Zamboni and Vendramini (1947). *Tra storte e provette*, p. 11. Milano.

Brit. J. Pharmacol. (1951), **6**, 185.

2:4-DIAMINOPYRIMIDINES—A NEW SERIES OF ANTIMALARIALS

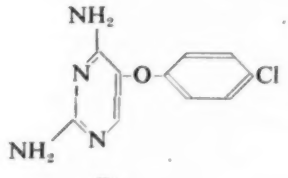
BY

E. A. FALCO, L. G. GOODWIN, G. H. HITCHINGS, I. M. ROLLO, AND P. B. RUSSELL

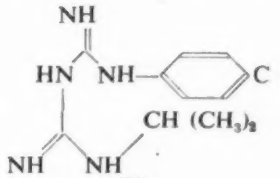
From the Wellcome Research Laboratories, Tuckahoe, New York, U.S.A., and the Wellcome Laboratories of Tropical Medicine, 183, Euston Road, London, N.W.1

(Received January 1, 1951)

It was found by Hitchings, Elion, VanderWerff, and Falco (1948) that many 2:4-diaminopyrimidines are powerful antagonists of pteroylglutamic acid in cultures of *Lactobacillus casei* (cf. Hitchings, Elion, Falco, Russell, Sherwood, and VanderWerff, 1950). The formal analogy between 2:4-diamino-5-*p*-chlorophenoxypyrimidine (I) and proguanil (" Paludrine ") (II), and the finding that proguanil was also an antagonist of pteroylglutamic acid, suggested that the pyrimidine compound might have antimalarial activity (Falco, Hitchings, Russell, and VanderWerff, 1949).



(I)



(II)

This was shown to be the case, and a short report of the antimalarial activity of another member of this group of compounds was published later by Goodwin (1949) A large number of derivatives of 2:4-diaminopyrimidine substituted in the 5- and 6-positions has now been prepared and tested against laboratory plasmodial infections. The results obtained upon 158 of these substances are recorded in the present paper.

METHODS

Preliminary " screening " tests were carried out against blood-induced infections of *Plasmodium gallinaceum* in chicks and *P. berghei* infections in mice. The more active substances were then assayed against standard antimalarials such as proguanil and quinine. Further tests were carried out with selected compounds against sporozoite-induced infections of *P. gallinaceum* in chicks and against trophozoite and sporozoite infections of *P. cynomolgi* in monkeys.

Acute and chronic toxicity tests and full pharmacological investigations have been made with the more active compounds, and will form the substance of a separate report.

Experimental details

Most of the pyrimidine derivatives were prepared for administration by dissolving the base in water with the addition of lactic acid. One or two compounds were administered as hydrochlorides, and the few which did not form readily soluble salts were suspended in water with the aid of compound powder of tragacanth.

P. gallinaceum *infections.*—The chicks used for the test were Light Sussex X Rhode Island cocks, and were hatched at the Reading University Farm at Shinfield, Berks.

Blood-induced infections.—The method used was similar to that of Davey (1946a). Five-day-old chicks were injected intravenously with 0.2 ml. of a suspension containing approximately 50×10^6 parasitized red cells, the first dose of drug was given orally 2–3 hours later, and further doses twice daily for 3 days. In preparing the inoculum the nomogram devised by Williamson (1948) proved useful and time-saving. Blood films were made at the peak of infection of control birds on the fifth day. Each compound was awarded a "score" according to the following key:

0 = No activity
1 = Slight activity
} at 100 mg./kg. in chicks or 50 mg./kg. in mice.
2 = Active at dose levels between 100 and 10 mg./kg.
3 = " " " " " 10 and 1 "
4 = " " " " " 1 and 0.1 "
5 = " " " " " 0.1 and 0.01 "

A dose was considered to be "active" when the mean percentage parasitaemia of the treated group of animals was lowered to 1 per cent or less of the mean percentage parasitaemia of untreated controls.

Active compounds were assayed by the method described by Marshall (1945). The mean percentage parasitaemia in each group of chicks was plotted against the logarithm of the dose, and a curve for each compound was drawn. A curve was determined for a standard antimalarial drug in the same experiment, and the relative activity of the compounds estimated from the ratio of the doses which effected a 50 per cent reduction in parasitaemia compared with untreated controls.

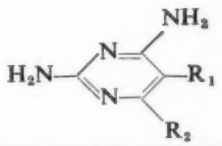
Sporozoite infections were induced by the method of Davey (1946b). Five-day-old chicks were inoculated intravenously with 0.2-ml. quantities of a suspension of sporozoites in a mixture of equal parts of 0.5 per cent glucose-saline and heparinized chick-blood. The suspension was prepared by grinding in a mortar the requisite number of lightly anaesthetized infected *Aedes aegypti* mosquitoes with a little of the saline-blood medium. The suspension was lightly centrifuged and the supernatant sporozoite suspension diluted with medium so that 0.2 ml. contained the equivalent of one mosquito. In untreated controls this inoculum usually caused death from blockage of the brain capillaries with exoerythrocytic schizonts in 9–10 days. Drug treatment was similar to that used for blood infections.

P. berghei *infections.*—The mice used for the test weighed from 20 to 30 grammes. The strain of plasmodium was that described by Vincke and Lips (1948), and was obtained through the kindness of Professor H. E. Shortt. The method used was similar to that described by Goodwin (1949), the mice being infected by intraperitoneal injection of approximately 5×10^6 parasitized red cells suspended in glucose-saline. The first dose of drug was given by mouth 2–3 hours after inoculation and further doses were given twice daily for 3 days. Blood films were made on the seventh day.

Attempts were made to cultivate *Anopheles concolor*, the vector of this parasite (Vincke and Leleup, 1949). Eggs and larvae were kindly sent from the Belgian Congo by Drs.

Vincke and Hanse, but we were unable to obtain the correct conditions for their development. For this reason, no results can as yet be recorded for the effect of the new compounds upon sporozoite-induced infections with *P. berghei*. Tissue-forms of the parasite have been described by Van den Berghe, Vincke, and Chardome (1950) in the histiocytes of the liver and the endothelial cells of the brain capillaries 36 hours after inoculation of infected blood, but we have not yet satisfied ourselves that the exoerythrocytic forms occur in mice after blood inoculation.

TABLE I

2: 4-Diamino-5-phenylpyrimidines 

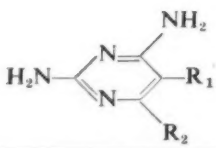
Reference No.	R_1	R_2	Score of activity; proguanil equivalents in parentheses	
			P. gallinaceum	*P. berghei*
48–187	phenyl	H	2	2
50–101	4′-methylphenyl	H	2	2
50–100	4′-methoxyphenyl	H	3	2
50–103	4′-nitrophenyl	H	2	2
50–82	2′-chlorophenyl	H	1	2
50–26	3′-chlorophenyl	H	2 (0.3 P)	2 (0.5 P)
49–248	4′-chlorophenyl	H	3 (0.4 P)	4 (30 P)
49–370	4′-bromophenyl	H	3 (0.6 P)	4 (10 P)
50–308	4′-fluorophenyl	H	3 (0.6 P)	4 (7 P)
50–59	phenyl	Me	3	2
50–200	phenyl	Et	3	2
50–201	4′-methylphenyl	Me	2	0
50–143	2′-chlorophenyl	Me	2	2
50–338	3′-fluorophenyl	Me	3	3
50–142	3′-chlorophenyl	Me	3	3
50–329	3′-bromophenyl	Me	3	3
50–283	4′-fluorophenyl	Me	4	3
50–58	4′-chlorophenyl	Me	4 (15 P)	4 (40 P)
50–322	4′-bromophenyl	Me	4	4
50–307	4′-fluorophenyl	Et	4 (5 P)	4 (7 P)
50–63	4′-chlorophenyl	Et	5 (60 P)	5 (200 P)
50–238	4′-bromophenyl	Et	4 (30 P)	4 (80 P)
50–148	4′-chlorophenyl	*n*-Pr	4 (20 P)	3 (5 P)
50–172	4′-chlorophenyl	*n*-Bu	4 (7 P)	3 (1 P)
50–146	4′-chlorophenyl	*iso*-Bu	4 (10 P)	3 (3 P)
50–109	4′-chlorophenyl	*n*-Am	4 (40 P)	3 (8 P)
50–256	4′-chlorophenyl	*n*-hexyl	4 (5 P)	3 (2 P)
50–251	4′-chlorophenyl	*n*-heptyl	4 (5 P)	3 (6 P)
50–141	4′-chlorophenyl	*n*-undecyl	3 (4 P)	3 (9 P)
50–115	4′-chlorophenyl	phenyl	3	2
50–230	4′-chlorophenyl	3-pyridyl	3 (0.5 P)	1
50–339	4′-chlorophenyl	4-pyridyl	2	0
50–198	4′-chlorophenyl	methoxymethyl	3	0
50–126	4′-nitrophenyl	Me	3 (2 P)	3 (2 P)
50–199	4′-nitrophenyl	Et	4 (4 P)	3
50–99	2′: 4′-dichlorophenyl	H	2	3 (1 P)
50–11	3′: 4′-dichlorophenyl	H	3 (0.7 P)	3
50–197	3′: 4′-dichlorophenyl	Me	4 (14 P)	5 (130 P)
50–276	3′: 4′-dichlorophenyl	Et	4 (20 P)	5 (190 P)
50–280	3′: 4′-dichlorophenyl	*n*-Pr	4	4
50–292	3′: 4′-dichlorophenyl	*n*-Bu	4	3

P. cynomolgi *infections*.—We are also indebted to Professor H. E. Shortt for trophozoite- and sporozoite-inoculated monkeys. Trophozoite infections were induced by the intraperitoneal injection of infected citrated blood. For infection with sporozoites, monkeys under pentobarbitone anaesthesia were placed in a cage containing a large number of infected mosquitoes which were allowed to feed. In neither trophozoite nor sporozoite infections was there any evaluation of the number of parasites inoculated. The monkeys were dosed orally with drugs, and stained thick and thin blood films prepared and examined at frequent intervals. Four animals with established, long-standing sporozoite infections were selected for investigation of the effect of prolonged treatment with large doses of three of the more active compounds. This experiment also served as a chronic toxicity test, and determinations of haemoglobin and red and white cell counts were made at intervals.

Toxicity tests.—Toxicity determinations were made in mice with some of the more active compounds and with a series of well-known antimalarial drugs. Two dose-levels were used for each compound, with a group of 10 mice at each level. Doses of drug were given by mouth, in solution or suspension in a volume of 0.5 ml. per 20 g. body weight

TABLE II

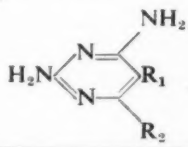
2: 4-Diamino-5-benzylpyrimidines



Reference No.	R_1	R_2	Score of activity; proguanil equivalents in parentheses	
			P. gallinaceum	*P. berghei*
48–224	benzyl	H	2	2
49–294	4′-methylbenzyl	H	2	0
49–169	2′-chlorobenzyl	H	1	2
48–257	4′-chlorobenzyl	H	2	2 (0.6 P)
50–62	4′-nitrobenzyl	H	3 (0.6 P)	2
49–161	4′-methoxybenzyl	H	2 (0.2 P)	2
49–210	3′: 4′-dimethoxybenzyl	H	3	1
49–267	4′-dimethylaminobenzyl	H	2 (0.15 P)	0
48–228	benzyl	Me	2	3
49–172	2′-chlorobenzyl	Me	2	2
49–224	4′-chlorobenzyl	Me	3 (0.4 P)	3 (2 P)
49–315	4′-bromobenzyl	Me	3 (0.3 P)	3 (1 P)
50–203	4′-chlorobenzyl	Et	3	2
49–336	4′-chlorobenzyl	*n*-Pr	2	1
49–302	3′-methylbenzyl	Me	2	0
49–371	4′-nitrobenzyl	Me	3 (1 P)	3 (1.5 P)
50–88	4′-aminobenzyl	Me	2	0
49–291	4′-methoxybenzyl	Me	2	1
49–292	2′: 4′-dichlorobenzyl	Me	2	3
50–61	3′: 4′-dichlorobenzyl	Me	2	2
50–2	3′: 4′-dimethoxybenzyl	Me	2 (0.2 P)	0
49–299	3′: 4′-methylenedioxybenzyl	Me	2	0
50–110	4′-acetamidobenzyl	Me	3	1
48–251	benzyl	phenyl	0	1
49–223	benzyl	Cl	0	0
48–243	benzyl	NH_2	2 (0.05 P)	1 (0.1 P)
49–295	4′-chlorobenzyl	NH_2	2	2

TABLE III

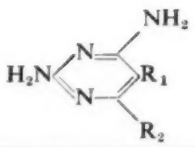
2: 4-Diamino-5-alkoxy- and -aryloxy-pyrimidines



Reference No.	R_1	R_2	Score of activity; proguanil equivalents in parentheses	
			P. gallinaceum	*P. berghei*
49–243	methoxy	H	0	0
48–211	ethoxy	H	2 (0.1 P)	1
49–251	*n*-butoxy	H	2 (0 2 P)	1 (0.3 P)
50–174	*n*-octyloxy	H	2	0
49–249	benzyloxy	H	2	2 (0.8 P)
49–200	phenoxy	H	2	1
49–173	4′-methylphenoxy	H	0	1
49–220	4′-tertbutylphenoxy	H	1	1
49–155	4′-*iso*-octylphenoxy	H	0	0
49–269	3′: 4′-dimethylphenoxy	H	1	0
49–272	2′-*iso*propyl-5′-methylphenoxy	H	0	0
49–69	α-naphthoxy	H	1	1
48–256	β-naphthoxy	H	2	2
49–170	β-naphthoxy	phenyl	1	0
49–74	6′-bromo-β-naphthoxy	H	1	1
49–70	2′: 6′-dichloro-α-naphthoxy	H	0	2
48–284	4′-phenylphenoxy	H	2	3 (1 P)
48–285	4′-benzylphenoxy	H	2	2 (1 P)
49–231	4′-hydrindylphenoxy	H	3	2
49–72	4′-hydroxyphenoxy	H	1	1
49–209	3′-methoxyphenoxy	H	1	1
49–229	4′-methoxyphenoxy	H	2	0
49–369	2′: 6′-dimethoxyphenoxy	H	0	0
49–42	4′-benzyloxyphenoxy	H	1	0
48–221	2′-chlorophenoxy	H	1	1
48–287	3′-chlorophenoxy	H	2 (0.05 P)	1
48–122	4′-chlorophenoxy	H	2	2
49–277	4′-bromophenoxy	H	0	1
48–152	2′: 4′-dichlorophenoxy	H	0	1
49–19	2′: 4′-dibromophenoxy	H	0	3
49–225	2′: 4′: 5′-trichlorophenoxy	H	0	0
49–160	4′-chloro-3′-methylphenoxy	H	1	0
49–226	4′-tertbutyl-2′-chlorophenoxy	H	1	0
49–159	4′-chloro-2′-phenylphenoxy	H	0	0
49–171	4′-chloro-5′-methyl-2′-*iso*propyl phenoxy	H	1	2
49–284	phenoxy	Me	2	1
50–104	4′-propionylphenoxy	Me	3	1
49–318	3′: 4′-dimethylphenoxy	Me	0	0
49–273	4′-methoxyphenoxy	Me	2	0
50–1	4′-phenylphenoxy	Me	2	2 (0.4 P)
49–142	β-naphthoxy	Me	0	1
49–175	β-naphthoxy	*n*-Pr	1	0
49–265	3′-chlorophenoxy	Me	3 (0.5 P)	1
48–210	4′-chlorophenoxy	Me	3 (0.4 P)	3 (0.7 P)
50–193	4′-chlorophenoxy	Et	3	1
50–78	4′-chlorophenoxy	*n*-Pr	2	1
49–233	4′-chlorophenoxy	phenyl	0	1
50–57	4′-nitrophenoxy	Me	3 (2 P)	3
50–73	4′-aminophenoxy	Me	2	0
50–87	4′-acetamidophenoxy	Me	1	1
50–167	4′-(*p*-chlorobenzyloxy) phenoxy	Me	2	1
50–183	4′-benzenesulphonylphenoxy	Me	1	0

TABLE IV

Miscellaneous derivatives of 2 : 4-diaminopyrimidine



Reference No.	R_1	R_2	Score of activity	
			P. gallinaceum	*P. berghei*
50–117	α-naphthyl	H	0	0
50–330	β-phenoxyethyl	H	0	0
50–346	β-phenoxyethyl	Me	1	0
49–250	nitro	H	0	0
49–376	aminomethyl	H	1	1
49–68	formylamino	H	0	0
49–92	chloroacetylamino	H	0	0
48–124	H	phenyl	0	0
49–61	H	β-naphthyl	0	0
48–29	Me	Me	2	0
48–174	*n*-propyl	Me	0	1
50–202	2-furyl	Me	1	1
50–249	2-thienyl	Me	1	0
50–305	dibromothienyl-2-	Me	3	4
50–173	2′-phenylthiazolyl-4-	Me	2	0
50–171	α-4′-chlorophenethyl	Me	2	0
48–98	bromo	Me	0	0
48–244	*n*-butyl	amino	2	1
49–8	Me	4′-chlorophenyl	0	0
Methiodides				
50–186	4′-chlorophenyl	H	0	1
50–284	4′-chlorophenoxy	Me	2	2

Results and Discussion

The results are presented in Tables I–IX. The activity of each compound is recorded as a " score " from 0 to 5 according to the key on p. 186, and the proguanil equivalents of the more potent substances are also recorded in parentheses.

Activity against blood-induced infections of P. gallinaceum *and* P. berghei

With a large series of substances such as this, which are closely related chemically and many of which have similar solubilities, it is profitable to consider the effect of structure upon therapeutic potency.

The effect of the 5-substituent.—Although some antimalarial activity is shown by substances with simple substituents in the 5-position of the pyrimidine nucleus (Table III, 48–211, 49–251; Table IV, 48–29) the really potent substances are found among those with substituents containing a benzene nucleus. Of these, the phenyl series (Table I) is generally more active than the benzyl series (Table II), which in turn is more active than the phenoxy series (Table III).

An electron-attracting group, such as nitro- or halogen in the *para-* position of the benzene ring enhances activity; *ortho-* and *meta-* derivatives are generally less active than the corresponding *para-* compounds (Table I, 50–82, 50–26, 49–248; Table II, 49–172, 49–224), although in the phenoxy series the *m*-chloro- compounds are about equal in activity to the *p*-chloro compounds against *P. gallinaceum* (Table III, 48–287, 48–122, 49–265, and 48–210). Corresponding chloro-, bromo-, and fluoro- compounds are all of about the same degree of activity when assessed by the

TABLE V

Miscellaneous pyrimidine derivatives and related compounds

Reference No.	Substance	Score of activity	
		P. gallinaceum	*P. berghei*
50–27	4-amino-5-phenylpyrimidine	1	0
50–28	4-amino-5-*p*-chlorophenylpyrimidine	2	0
50–66	4-amino-2-methyl-5-*p*-chlorophenyl pyrimidine	0	0
50–67	4-amino-2: 6-dimethyl-5-*p*-chlorophenyl pyrimidine	1	0
50–125	2-amino-4-hydroxy-5-*p*-chlorophenyl pyrimidine	2	0
48–231	2-amino-4-hydroxy-6-methyl-5-*p*-chlorophenoxy-pyrimidine	1	0
48–194	2-amino-4-mercapto-5-*p*-chlorophenoxy pyrimidine	0	1
50–325	2: 4-*bis* (N-morpholino)-6-phenylpyrimidine	0	0
50–79	4-amino-2-*p*-methylphenyl-5-*p*-chlorophenyl pyrimidine	1	0
49–128	2-amino-4-methylamino-5-*p*-chlorophenoxy pyrimidine	2	1
50–187	2-amino-4-methyl-6-(6′-aminoquinolyl)-pyrimidine	0	0
48–204	2: 4-diamino-6-chloroquinazoline	0	1
49–122	2: 4-diamino-8-*p*-chlorophenylpurine	0	0
49–373	2: 4-diamino-6: 7-di(*p*-chlorophenyl)-pteridine	0	2
49–274	2: 4-diamino-6 or 7-*o*-chlorophenyl-7 or 6-*p*-methoxyphenyl-pteridine	2	1
48–14	2: 6-diaminopurine	1	1
45–70	5-hydroxyuracil	0	1

key on p. 186, but accurate determination of the relative potencies of these derivatives shows that the fluoro- compounds are always slightly less active than the chloro- and bromo- compounds (Fig. 1).

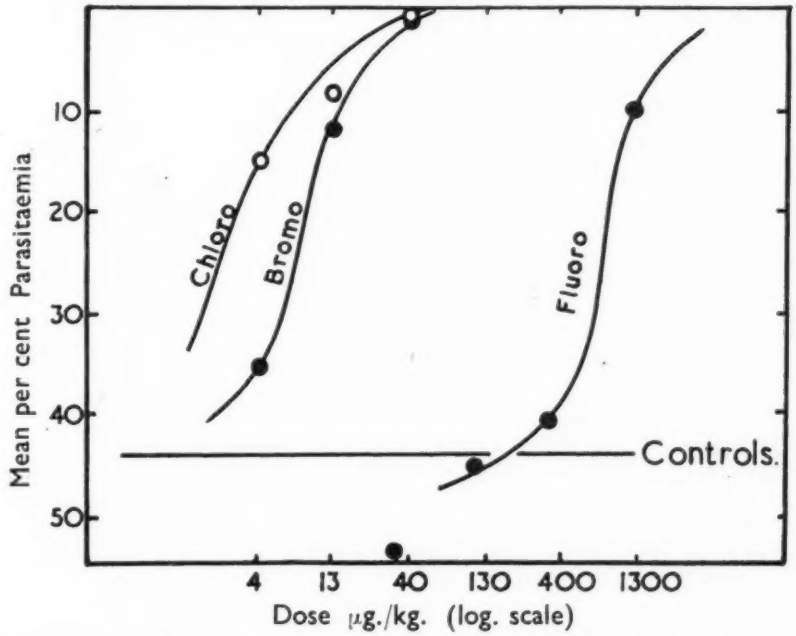


FIG. 1.—A comparison of the activities against *P. berghei* of 2: 4-diamino-5-*p*-chlorophenyl-6-ethyl pyrimidine (50–63) and the corresponding bromo- and fluoro- derivatives. All administered by mouth as solutions of the lactates; a group of 5 mice used at each dose-level.

Dichloro- derivatives in the phenyl series (Table I, 50–99, 50–11, 50–197, 50–276, 50–280, 50–292) are also very potent antimalarials especially against *P. berghei*, and have activities approximately equal to the corresponding *p*-chloro- compounds. The 3′: 4′-dichlorophenyl-6-methyl compound (50–197), although about equal in activity to the corresponding 4′-chlorophenyl compound (50–58) against *P. gallinaceum*, is actually three times as active against *P. berghei*. The 3′: 4′-dichlorophenyl-6-ethyl compound (50–276) has one-third of the activity of the corresponding 4′-chlorophenyl compound (50–63) against *P. gallinaceum*, but the two substances are equally active against *P. berghei*. Phenoxy derivatives with more than one halogen substituent, on the other hand, are almost without action (Table III, 48–152, 49–19, 49–225). Toxicity tests show that the 3′: 4′-dichlorophenyl compounds are rather more toxic than the monochloro-, and delayed deaths are found to occur among the mice for 5 days after oral administration of the drug (Table VI). A similar prolonged toxic action was observed with the *p*-chlorophenyl compound with no 6-substituent (Table I, 49–248).

The failure of a second chlorine atom in the benzene ring to enhance activity against *P. gallinaceum* in the 2: 4-diaminopyrimidines is interesting in view of the recent publication of Curd, Davey, Hendry, and Rose (1950), in which the dichloro-derivative corresponding to proguanil is shown to be 2.5 to 5 times as active as proguanil. It is also more toxic to some species of animals. We find this compound in preliminary tests to be 1 to 3 times as active as proguanil against *P. berghei*.

The nitro- compounds of the phenyl series are less active than the corresponding chloro- compounds; in the phenoxy and benzyl series the nitro- derivatives are somewhat more active than the chloro-, especially when *P. gallinaceum* is used as the test-organism.

Electron-donating substituents such as alkyl and methoxy groups do not increase antimalarial activity (Table I, 50–101, 50–100).

Alkoxy compounds above methoxy (Table III, 48–211, 49–251, 50–174) have roughly the order of activity of the unsubstituted phenoxy derivatives. However, by appropriate substitutions the activity of the latter may be greatly enhanced, whereas the alkoxy series is not subject to similar modification.

The effect of the 6-substituent.—The antimalarial activity of the *p*-chloro derivatives is enhanced by the introduction of a methyl group in the 6-position of the pyrimidine nucleus. The same generalization probably applies to compounds with other electron-attractive substituents in the benzene nucleus, but does not apply to the dimethylphenoxy or naphthoxy derivatives (Table III, 49–269, 49–318, 48–256, 49–142). When a series of alkyl derivatives was compared the methyl derivative was found to be the most active in both the benzyl (Table II, 49–224, 50–203, 49–336) and phenoxy series (Table III, 48–210, 50–193, 50–78). The effect of the nature and length of the alkyl radical was studied in considerable detail in the phenyl series, in which the peak of activity is reached with the 6-ethyl compound, 50–63 (Table I), which is 60 times as active as proguanil against *P. gallinaceum*, and 200 times as active against *P. berghei*. Higher and lower members of the series are less active, but to a different degree with the two species of plasmodium. *P. berghei* is rather less sensitive than *P. gallinaceum* to the higher members of the series, but these differences may perhaps be explained by differences in the rates of absorption,

metabolism, and excretion of the drugs by the mammalian and avian hosts. In this series it is also noteworthy that the *iso*butyl is more active than the *n*-butyl derivative, and that there is a tendency for compounds with an odd number of C-atoms in the chain to be more active than compounds with an even number of C-atoms. The relative activities of this group of compounds (Table I, 49–248, 50–58, 50–63, 50–148, 50–172, 50–109, 50–256, 50–251, 50–141) are shown diagrammatically in Fig. 2.

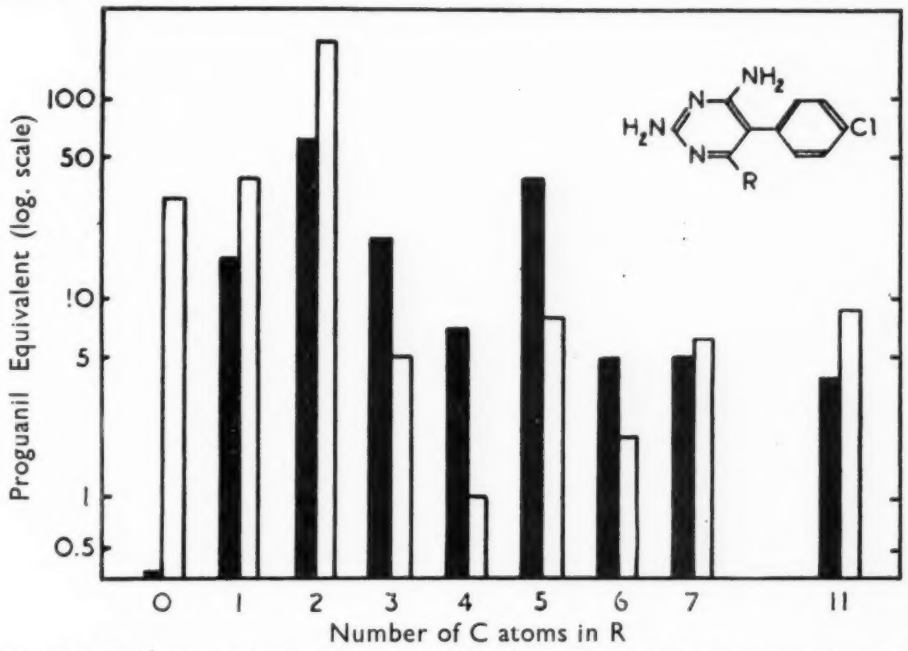


FIG. 2.—The effect upon antimalarial activity of the chain length of the alkyl group in a series of 2: 4-diamino-5-*p*-chlorophenyl-6-alkyl-pyrimidines. Open columns: *P. berghei*; solid columns: *P. gallinaceum*.

The effects of substituents of other types in the 6-position do not lend themselves to ready interpretation. The 6-phenyl derivatives are less active than their alkyl congeners (Table I, 50–115; Table II, 48–251; Table III, 49–233). A 3-pyridyl derivative (Table I, 50–230) has approximately the activity of the phenyl, but the 4-pyridyl is much less active (Table I, 50–339). In the benzyl series the 6-amino derivatives are only slightly less active than the compounds unsubstituted in position 6, but the 6-chloro- compound is essentially inactive (Table II, 48–243, 49–295, 49–223).

Quaternization of a pyrimidine-N atom greatly decreases antimalarial activity (Table IV, 50–186, 50–284).

The relationship of the 2: 4-diaminopyrimidines to similar compounds differing by substitution of the amino groups is worthy of consideration. A large series of pyrimidine derivatives with alkylaminoalkylamino side chains was studied by Davey (1946a) and Curd, Richardson, and Rose (1946). On the basis of their results and those reported in the present paper, at least two types of pyrimidine antimalarials appear to be distinguishable. The first type is exemplified by those pyrimidines carrying phenyl, phenoxy, or benzyl groupings in the 5-position, and in which

substitution of the 2- and/or 4-amino groups appears to diminish the antimalarial activity. For example, 2-*p*-chloroanilino-4-β-diethylaminoethylaminopyrimidine (Curd, Richardson, and Rose, 1946) is less active than 2-amino-4-β-diethylaminoethylaminopyrimidine (Hull, Lovell, Openshaw, and Todd, 1947), which in turn is less active than 2: 4-diamino-5-phenoxypyrimidine (Table III, 49–200). A similar stepwise effect does not appear to be true of the derivatives of 5-benzyl-6-methylpyrimidine, but it is abundantly clear that the unsubstituted 2: 4-diamino-5-benzyl-6-methylpyrimidine (Table II, 48–228) is much more active than the corresponding compounds with substituted amino groups (Hull, Lovell, Openshaw, Payman, and Todd, 1946; Curd, Richardson, and Rose, 1946) and that the 2: 4-diamino-5-phenylpyrimidine (Table I, 48–187) is much more active than related amino-substituted derivatives (Curd, Richardson, and Rose, 1946). Similarly a 2-amino-4-methylamino-5-*p*-chlorophenoxypyrimidine (Table V, 49–128) is less active than the corresponding 2: 4-diamino derivative (Table III, 48–122).

TABLE VI

Acute oral toxicities in mice and antimalarial activities of selected 2: 4-diaminopyrimidines compared with standard antimalarial drugs

Drug	LD50 (mg./kg.)	Limits of error per cent (P = 0.99)	Time taken for mice to die	Proguanil equivalent against *P. gallinaceum*	Proguanil equivalent against *P. berghei*
Quinine HCl	1,160	91–111	1 hr.	0.08	0.16
Proguanil acetate	59	83–121	24 hr.	1.0	1.0
Mepacrine HCl	871	82–123	20 min.	0.1	1.7
Chloroquin base	752	88–114	15 min.	1.2	3.3
Pamaquin B.P.	68	85–118	1 hr.	1.2	0.53
2: 4-Diamino-5-(4′-chlorophenoxy)-6-methylpyrimidine (48–210)	ca. 1,000	—	24 hr.	0.4	0.7
2: 4-Diamino-5-(4′-chlorobenzyl)-6-methylpyrimidine (49–224)	79	83–113	½ hr.	0.4	2.0
2: 4-Diamino-5-(4′-chlorophenyl)-pyrimidine (49–248)	250	55–180	Up to 3 weeks	0.4	30
2: 4-Diamino-5-(4′-nitrobenzyl)-6-methylpyrimidine (49–371)	146	93–101	3–4 hr.	1.0	1.5
2: 4-Diamino-5-(4′-chlorophenyl)-6-ethylpyrimidine (50–63)	92	94–108	10 min.	60	200
2: 4-Diamino-5-(3′: 4′-dichlorophenyl)-6-ethylpyrimidine (50–276)	66	82–120	Up to 5 days	20	190

The second type comprises pyrimidines which are unsubstituted, or carry only alkylsubstituents in the 5-position (those which eventually led to the more active acyclic analogue, proguanil). In this group of compounds, substitution of the amino group is requisite to activity (Basford, Curd, Hoggarth, and Rose, 1947). This difference between the two groups appears to be borne out by the reported absence of cross-resistance between diaminopyrimidines and proguanil which is discussed below.

The essential nature of the 2- and 4-amino group in the pyrimidine nucleus for activity in the present series receives further confirmation from the lower activity

of 2-amino-4-hydroxy (Table V, 50–125, 48–231) and 2-amino-4-mercapto (Table V, 48–194) derivatives as compared with the corresponding 2: 4-diaminopyrimidines (Table III, 48–210, 48–122). Furthermore, 4-amino-5-phenyl- and 4-amino-5-*p*-chlorophenylpyrimidines (Table V, 50–27 and 50–28) are only feebly active as compared with the corresponding diamino-derivatives (Table I, 48–187, 49–248). A number of condensed systems containing the 2: 4-diaminopyrimidine structure show moderate though generally unpromising activity (Table V, 48–204, 48–14). In this category the pteridines (Table V, 49–373, 49–274) possess activities similar to those of the 2: 4-diamino-pteridines studied by Greenberg (1949).

Acute oral toxicities in mice of some of the more active compounds from the three main series are shown in Table VI in comparison with those of standard antimalarial drugs.

TABLE VII

The effect of 2 : 4-diaminopyrimidines upon heavy sporozoite-induced infections of *P. gallinaceum* (1 mosquito per chick)

Reference No.	Substituents	Dose mg./kg.	No. of chicks	No. protected for 28 days	No. living longer than controls	No. showing E.E. forms *post mortem*
Proguanil	—	50	(toxic)			
		40	5	1	2	2
		25	6	0	6	6
		20	10	4	2	3
		12.5	6	0	3	4
		10	5	1	0	2
		5	4	1	0	2
48–210	5-*p*-chlorophenoxy-6-methyl	40	5	1	4	1
		32	6	0	6	6
		20	10	3	8	3
		16	6	0	5	5
		10	5	2	2	3
		8	6	0	5	6
		5	5	0	2	3
49–224	5-*p*-chlorobenzyl-6-methyl	50	(toxic)			
		40	4	2	2	0
		25	6	2	4	3
		20	5	2	3	0
49–248	5-*p*-chlorophenyl	80	(toxic)			
		40	9	2	7	6
		20	9	3	7	5
49–371	5-*p*-nitrobenzyl-6-methyl	40 (toxic to 4/6)	6	2	2	0
		20 (toxic to 3/6)	6	2	3	1
48–211	5-ethoxy	20	5	1	1	3
		10	5	1	1	3
		5	5	0	0	5
49–161	5-*p*-methoxybenzyl	40	5	1	3	4
		20	5	0	1	5

Sporozoite-induced infections of P. gallinaceum *and* P. cynomolgi

The results of treatment of sporozoite-induced infections of *P. gallinaceum* in chicks with a selection of substances are shown in Tables VII and VIII. It is apparent that the 2: 4-diaminopyrimidines are at least as efficient as proguanil in delaying death from the development of exoerythrocytic schizonts. Greenberg, Trembley, and Coatney (1950) have recently demonstrated that very heavy sporozoite infections such as were used in the experiments recorded in Table VII may give a false impression of the value of antimalarial drugs in the treatment of human malaria. They showed that the results obtained by Alving *et al.* (1948) in natural

TABLE VIII

The effect of 2 : 4-diaminopyrimidines upon heavy and light sporozoite infections of *P. gallinaceum*

Inoculum	Reference No.	Substituents	Dose mg./kg.	No. of chicks	No. protected for 28 days	No. living longer than controls	No. showing E.E. forms *post mortem*
Heavy (One mosquito per chick)	Proguanil		30	6	0	3	3
			15	6	0	3	3
	49–224	5-*p*-chlorobenzyl-6-methyl	25	6	1	2	0
			10	5	1	4	3
	50–58	5-*p*-chlorophenyl-6-methyl	5	(Toxic)			
			2	6	1	1	0
	50–63	5-*p*-chlorophenyl-6-ethyl	5* Toxic to 5/6 chicks		1	1	0
			2	6	1	1	0
	50–109	5-*p*-chlorophenyl-6-*n*-amyl	5*	6	2	4	0
			2	6	3	4	0
	50–141	5-*p*-chlorophenyl-6-*n*-undecyl	5*	6	0	4	4
			2	4	0	0	2
Light (0.01 mosquito per chick)	Proguanil		30	6	1	2	0
			15	5	2	3	1
	49–224	5-*p*-chlorobenzyl-6-methyl	25	6	0	0	0
			10	6	1	1	0
	50–58	5-*p*-chlorophenyl-6-methyl	5*	6	3	3	0
			2	6	2	2	0
	50–63	5-*p*-chlorophenyl-6-ethyl	5*	5	2	2	0
			2	5	1	2	0
	50–109	5-*p*-chlorophenyl-6-*n*-amyl	5*	6	0	0	0
			2	6	2	2	0
	50–141	5-*p*-chlorophenyl-6-*n*-undecyl	5*	6	0	1	3
			2	6	0	0	4

*Dose given once daily.

TABLE IX

The effect of three 2 : 4-diaminopyrimidines upon *P. cynomolgi* infections in monkeys. Substituents:—49–224: 5-*p*-chlorobenzyl-6-methyl; 49–248: 5-*p*-chlorophenyl; 50–63: 5-*p*-chlorophenyl-6-ethyl

Method of infection	Monkey No.	Drug	Treatment	Result
(1) Blood inoculation	60	49–224	4 mg./kg. twice daily for 6½ days beginning on 10th day after inoculation	Blood became free from parasites on 6th day of treatment. No relapses followed within 8 weeks of splenectomy 20 days later. Conclusion: cured
	63	49–224	15 mg./kg. twice daily for 6½ days beginning on 10th day after inoculation	Blood became free from parasites on 6th day of treatment. No relapse followed within a week of splenectomy 20 days later. Conclusion: probably cured
	62	—	Control	Primary infection reached peak before 10th day and then decreased. Relapsed immediately after splenectomy and again 8 weeks later
(2) Sporozoite infection by mosquito bite	3	49–224	40 mg./kg. given on 4th day after infection	Parasites appeared in the blood 10 days after treatment. Conclusion: no effect upon pre-erythrocytic forms
			4 mg./kg. twice daily for 5 days beginning 14 days after infection	Blood freed from parasites, but a relapse occurred 4 weeks later. Conclusion: blood forms cleared, but no effect upon exoerythrocytic forms
	5	49–224	Two doses of 20 mg./kg. given 1½ hr. before infection	Parasites appeared in the blood 14 days after infection. Conclusion: no effect upon sporozoites
	4	49–248	4 mg./kg. twice daily for 5 days, beginning 14 days after infection	Parasites disappeared from the blood on 3rd day of treatment, but a relapse occurred 4 weeks later. Conclusion: blood forms cleared; no effect on E.E. forms
(3) Sporozoite infection by mosquito bite	11	50–63	5 mg./kg. twice daily for 4½ days beginning 7 days after infection	No parasites appeared until the 30th day. Conclusion: probably a suppressive action due to prolonged presence of the drug in the tissues
	12	—	Control	Parasites appeared in the blood 13 days after infection and persisted for 12 days

After a period of rest, monkeys 11, 5, and 3 were given long courses of substances 50–63, 49–371, and 48–210 respectively. In all, 57 doses each of 5 mg./kg. were given during 6 weeks. No parasites were found in the blood of any monkey during treatment or for 25 days afterwards. The 3 animals, and also monkey 12 to act as a control, were then splenectomized; all four relapsed 4 days later.

human malarial infections with pamaquin treatment were most closely paralleled in *P. gallinaceum* when they used very light infections produced by injecting the equivalent of 0.01 mosquito per chick. We have therefore compared treatment with 2: 4-diaminopyrimidines in heavy and light sporozoite infections, and the results are set forth in Table VIII.

It will be seen that with compounds 49–224, 50–58, 50–63, and 50–109, when the chicks are lightly infected, no exoerythrocytic forms could be found, although some of the birds failed to survive to the end of the test.

Table IX shows the results of treatment of a small number of rhesus monkeys infected with heavy inocula of sporozoites of *P. cynomolgi*. It will be seen that, although small doses of the drugs used were sufficient to suppress the infection, there was little effect upon the tissue forms of the parasite; even 5 mg./kg. given twice daily for six weeks did not prevent or delay the appearance of a heavy relapse of blood infection which occurred a few days after splenectomy.

In spite of the intensive treatment, no ill effects were observed. None of the animals refused food or showed any signs of toxic effects, and the haemoglobin and the red and white cell counts were unaltered. More extensive studies of the toxicity and pharmacology of the 2: 4-diaminopyrimidines will be reported in a further communication.

Action upon other organisms

Many of the compounds, including all the more active antimalarials, were tested against laboratory infections of *Trypanosoma rhodesiense*, *T. congolense*, *T. cruzi*, *Leishmania donovani*, and *Entamoeba histolytica*, by the methods described by Burn, Finney, and Goodwin (1950). No significant activity, apart from slight activity against *T. congolense* by the phenoxy derivatives, was shown by any. However, many of the 2: 4-diaminopyrimidines have powerful antibacterial activity *in vitro*. Individual compounds vary in the degree to which they are affected by the presence of blood and serum, and none has very great antibacterial activity *in vivo*. (Observations of Mr. H. VanderWerff and Mr. S. R. M. Bushby.) It is interesting that the more powerful antimalarials have, in general, the weaker antibacterial action; the best antibacterials are mostly compounds with electron-donating groups in the *para*- position of the 5-benzene nucleus. An important exception to this generalization is found in the phenyl series (Table I), in which the most active antimalarial (50–63) has also the highest antibacterial action. In both the phenoxy and benzyl series the compounds with H in the 6-position are more active than 6-alkyl derivatives against bacteria *in vitro*.

Possible use in human malaria

It may be valuable to speculate upon the possible uses of these compounds in human malaria. In view of the fact that they show very high activity against three species of *Plasmodium* in experimental animals, it is likely that they will also be of use against the parasites of man. The effective dose of the 5-*p*-chlorophenyl-6-ethyl derivative (50–63) is so small that it may well prove an economical compound for suppression, and, because of its relatively low toxicity, a potent drug for the treatment of overt infections. However, as a true causal prophylactic against *P. vivax* it is less likely to be of value, because large doses fail to affect the exoerythrocytic stages of *P. cynomolgi*.

A further consideration is the possibility of the value of these substances in the treatment of the proguanil-resistant strains of human malaria which are now appearing in several parts of the world (Edeson and Field, 1950). Greenberg and Richeson (1950) found that 2: 4-diamino-5-phenoxypyrimidines, like proguanil, acted synergistically with sulphadiazine upon *P. gallinaceum* infections, and therefore were likely to act upon the same metabolic systems of the parasite—probably those mediated by pteroylglutamic acid. However, the modes of action of the pyrimidines and proguanil may not be identical, as antagonism to pteroylglutamic acid and antimalarial activity do not run in parallel, and also a proguanil-resistant strain of *P. gallinaceum* does not show cross-resistance to 2: 4-diamino-5-*p*-chlorophenoxy-6-methylpyrimidine (48–210). Our own experience with drug-resistant strains of *P. gallinaceum* and *P. berghei* has confirmed and amplified these observations, and it is certain that the 2: 4-diaminopyrimidines are valuable in the treatment of proguanil-resistant strains. This work will be reported in detail in a later communication.

SUMMARY

1. A series of derivatives of 2: 4-diaminopyrimidine has been prepared and tested for antimalarial activity.

2. High activity is shown by many members against *P. gallinaceum* in chicks and *P. berghei* in mice.

3. Substances with a 5-phenyl substituent are the most active; 5-benzyl and 5-phenoxy derivatives are somewhat less active.

4. Substitution of halogen or nitro groups in the *para-* position of the benzene nucleus of the 5-substituent enhances activity.

5. Substitution of an alkyl group in the 6-position enhances activity, and, in the 5-phenyl derivatives, a peak of activity is reached with the 6-ethyl compound. 2: 4-Diamino-5-*p*-chlorophenyl-6-ethylpyrimidine is 60 times as active as proguanil against *P. gallinaceum* and 200 times as active against *P. berghei*. Longer chain alkyl derivatives are less active.

6. The drugs are also active against the blood-forms of *P. cynomolgi* in monkeys, but have no pronounced action upon the exoerythrocytic stages.

7. It is hoped that these drugs will prove to be of value in the suppression and treatment of human malaria, and especially in the treatment of proguanil-resistant strains.

We are indebted to Professor H. E. Shortt, F.R.S., for the strain of *P. berghei* and for monkeys infected with *P. cynomolgi*, and to Dr. I. H. Vincke and Dr. M. A. Hanse for eggs and larvae of *Anopheles concolor*. We also wish to thank Miss G. B. Elion for the purines and pteridines, Miss E. M. Beveridge and Dr. R. A. Neal for tests upon various protozoal infections, and Mr. H. VanderWerff and Mr. S. R. M. Bushby for microbiological studies. Miss S. DuBreuil and Miss P. Lee Graham have given valuable assistance in the preparation of the pyrimidines, and Miss J. Hughes, Miss G. Horton, and Mr. M. W. Cheeseman have been responsible for much of the routine work upon malarial infections. To all of these we are very grateful.

We also wish to acknowledge our indebtedness to Dr. C. H. Kellaway, F.R.S., and to Brig. J. S. K. Boyd for their counsel and for their work in facilitating the co-operation between the laboratories in New York and London.

REFERENCES

Alving, A. S., Pullman, T. N., Craige, B., jun., James, R., jun., Whorton, C. M., and Eichelberger, L. (1948). *J. clin. Invest.*, **27**, 34.
Basford, F. R., Curd, F. H. S., Hoggarth, E., and Rose, F. L. (1947). *J. chem. Soc.*, 1354.
Berghe, L. van den, Vincke, I. H., and Chardome, M. (1950). *Ann. Soc. belge Méd. trop.*, **30**, 79.
Burn, J. H., Finney, D. J., and Goodwin, L. G. (1950). *Biological Standardisation*, 2nd ed. London: Oxford University Press.
Curd, F. H. S., Davey, D. G., Hendry, J. A., and Rose, F. L. (1950). *Brit. J. Pharmacol.*, **5**, 438.
Curd, F. H. S., Richardson, D. N., and Rose, F. L. (1946). *J. chem. Soc.*, 378.
Davey, D. G. (1946a). *Ann. trop. Med. Parasit.*, **40**, 52.
Davey, D. G. (1946b). *Ann. trop. Med. Parasit.*, **40**, 453.
Edeson, J. F. B., and Field, J. W. (1950). *Brit. med. J.*, **1**, 147.
Falco, E. A., Hitchings, G. H., Russell, P. B., and VanderWerff, H. (1949). *Nature, Lond.*, **164**, 107.
Goodwin, L. G. (1949). *Nature, Lond.*, **164**, 1133.
Greenberg, J. (1949). *J. Pharmacol.*, **97**, 484.
Greenberg, J., and Richeson, E. M. (1950). *J. Pharmacol.*, **99**, 444.
Greenberg, J., Trembley, H. L., and Coatney, R. G. (1950). *Amer. J. Hyg.*, **51**, 194.
Hitchings, G. H., Elion, G. B., Falco, E. A., Russell, P. B., Sherwood, M. B., and VanderWerff, H. (1950). *J. biol. Chem.*, **183**, 1.
Hitchings, G. H., Elion, G. B., VanderWerff, H., and Falco, E. A. (1948). *J. biol. Chem.*, **174**, 765.
Hull, R., Lovell, B. J., Openshaw, H. T., Payman, L. C., and Todd, A. R. (1946). *J. chem. Soc.*, 357.
Hull, R., Lovell, B. J., Openshaw, H. T., and Todd, A. R. (1947). *J. chem. Soc.*, 41.
Marshall, P. B. (1945). *J. Pharmacol.*, **85**, 299.
Vincke, I. H., and Leleup, N. (1949). *Rev. Zool. Bot. Afr.*, **42**, 250.
Vincke, I. H., and Lips, M. (1948). *Ann. Soc. belge Méd. trop.*, **28**, 97.
Williamson, J. (1948). *Ann. trop. Med. Parasit.*, **42**, 238.

Note added in proof: Two of the compounds, 50–63 (Table I) and 49–224 (Table II), were further tested against blood-induced *P. cathemerium* infections of canaries. The minimum effective dose (MED), here defined as the dose which effected a 50 per cent reduction in parasitaemia compared with untreated controls, was estimated graphically as previously described from percentage parasitaemia counts on the sixth day.

The results are summarized in the Table below. The results with *P. gallinaceum* for 49–224 and 50–63 were estimated from previous assays and are the means of two or more experiments: the results for proguanil in both *P. gallinaceum* and *P. cathemerium* infections were taken from the paper by Davey (1946a).

Drug	*P. gallinaceum* MED	*P. cathemerium* MED	Ratio $\frac{\text{MED } P. \text{ cath.}}{\text{MED } P. \text{ gall.}}$
49–224	3.6 mg./kg. (mean of two)	>15 mg./kg.	>4
50–63	0.026 mg./kg. (mean of four)	0.2 mg./kg.	8
Proguanil ..	0.25 mg./50 g. (5 mg./kg.)	0.5 mg./20 g. (25 mg./kg.)	5

These results show that two members of the new series, previously shown to be highly active against *P. gallinaceum* and *P. berghei*, are considerably less effective against *P. cathemerium*. However, proguanil shows corresponding variations, yet is active against the plasmodia which infect man, and it may be expected that these two compounds act in a similar way.

Brit. J. Pharmacol. (1951), **6**, 201.

CYTOTOXIC AGENTS: I, METHYLOLAMIDES WITH TUMOUR INHIBITORY ACTIVITY, AND RELATED INACTIVE COMPOUNDS

BY

J. A. HENDRY AND F. L. ROSE

From Imperial Chemical Industries, Ltd., Research Laboratories, Hexagon House, Manchester, 9

AND

A. L. WALPOLE

From Imperial Chemical Industries, Ltd., Biological Laboratories, Wilmslow, Cheshire

(Received January 19, 1951)

In 1945 our colleagues Templeman and Sexton reported a study of the effects of arylcarbamic esters and related compounds upon the growth and morphology of cereal seedlings. They confirmed and extended the observations of Lefèvre (1939) that ethyl-N-phenylcarbamate produces bulbous hypertrophies of the coleoptile, the mesocotyl, and the root tip of wheat seedlings with progressive retardation or even arrest of growth. They found *iso*propyl-N-phenylcarbamate to be even more active in this sense and regarded the underlying cytotoxic effect of these substances as a blocking of mitosis in a pseudo-metaphase essentially similar to that produced by colchicine. Ethyl carbamate (urethane) was found to be inactive.

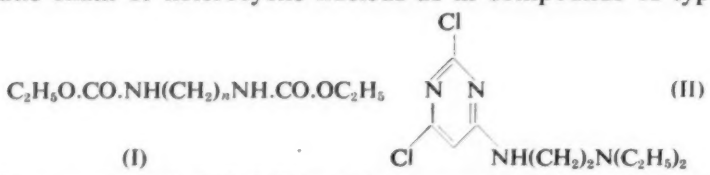
These findings prompted a study of the effects of the carbamates upon tumour growth in mammals. It was found (Haddow and Sexton, 1946) that both ethyl and *iso*propyl phenylcarbamate produce some retardation of the growth of the Walker carcinoma 256 in rats, but, somewhat unexpectedly, that urethane itself is even more active in this respect. These observations led ultimately to the clinical trial of urethane in advanced and intractable cancer in man and to the discovery that the drug causes striking though only temporary remissions in a high proportion of cases of myeloid and lymphatic leukaemia (Paterson, apThomas, Haddow, and Watkinson, 1946).

In the hope of finding a compound with an even more pronounced selective effect upon malignant tissues and at the same time of discovering some clue to the mode of action of urethane we prepared some 30 simple homologues of that substance and examined them for growth inhibitory activity against the Walker tumour. An account of this work is in preparation ; suffice it here to say that of those compounds only bis- and tris-carbethoxyamine even approach urethane in activity. From the results of similar studies both Haddow (private communication ; *v.* also Haddow and Sexton, 1946) and Skipper and his associates (Skipper *et al.*, 1948, 1949) have concluded that the effect of urethane upon tumours and upon leukaemic cells is shared by only a few substances very closely related to it.

In spite of the considerable amount of speculation and laboratory and clinical investigation that has been devoted to the problem, the mode of action of urethane remains obscure. The sequence of observations recorded here had as their starting point speculations upon this subject. Urethane is comparatively inert in the chemical sense but in some circumstances will react with amino groups according to the general equation:

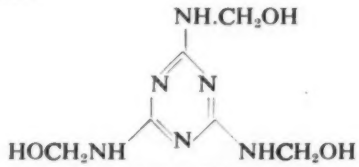
$$NH_2CO.CC_2H_5 + NH_2R \longrightarrow NH_2CO.NHR + C_2H_5OH$$

Although not facile *in vitro*, the reaction might occur *in vivo* under relatively mild conditions. We envisaged the possibility that urethane might interfere with cellular proliferation by reacting in this manner with some amino derivative within the cell essential to the process of division. In consequence we examined a range of substances formally capable of analogous interaction with amino groups to yield not only ureas and ureides as urethane does, but also other functional derivatives. These substances included organic halides, carboxylic esters, nitriles, cyanamides and dicyandiamides, cyanates, isocyanates, and isocyanate "generators," thiocyanates, ketones, aldehydes, imino-ethers, acetylenes, thiols, and alkylthiols. The majority were monofunctional in that the molecule carried only one reactive group, and all these were inactive against the Walker tumour, in daily doses near the maximum tolerated, in the sense that none produced any greater inhibition of tumour growth than could be accounted for on the basis of non-specific toxicity. A minority were polyfunctional, having two or more reactive groupings attached, for example, to an aliphatic chain or heterocyclic nucleus as in compounds of types I and II.



(I) (II)

These were included deliberately in consideration of the work of Gilman and Philips (1946) upon the biological effects of the alkyl nitrogen mustards. We had realized that the latter fell within the scope of our working hypothesis as substances capable of reacting with amino groups, and noted that in this series two alkylating groups appeared to be necessary for characteristic biological activity. It was in the polyfunctional class that tumour inhibitory activity was eventually encountered, namely, with compound (III) which was included for test in a series of polymethylolamides derived from amides by condensation with formaldehyde and characterized by their capacity to condense in turn with amines and other active hydrogen systems with the elimination of water.



(III) Trimethylolmelamine

Activity was subsequently found in several compounds related to (III).

The first polymethylolamides examined were obtained from the textile auxiliaries section of our research organization. When incorporated in fabrics derived from

natural or synthetic fibres these substances modify the mechanical properties of the fabrics in various ways, conferring, for example, increased wet strength upon paper and crease-resisting properties upon cotton or woollen textiles. Their usefulness in this direction is probably associated, as described by our colleague Mr. J. G. Evans (1949), with their poly-alkylating function and with the formation of cross-linkages between adjacent polymer fibres. The possible biological significance of the poly-alkylating and cross-linking potentialities of the sulphur and nitrogen mustards had been brought to our attention by Professor A. Haddow and his group (private communication, and Haddow, Kon, and Ross, 1948), more particularly in connexion with the aryl nitrogen mustards then under investigation by them ; this observation gave additional stimulus to the hypothetical ideas then being developed. The dependence of the characteristic toxicological properties of the nitrogen mustards upon the presence in the molecule of at least two β-chloroethyl groups emerges from early work with these agents and had been noted, for example, by Karnovsky, Burchenal, Ormsbee, Cornman, and Rhoads (1947) ; similarly Elmore, Gulland, Jordan, and Taylor (1948) found evidence for cross-linkage in one of the products isolated from the reaction between desoxyribonucleic acid and mustard gas, and suggested that their investigation provided a chemical parallel to previous cytological observations with the latter. While the methylolamides were under investigation we learnt also from Professor Haddow of the hypothesis, since elaborated and published (Goldacre, Loveless, and Ross, 1949 ; Loveless and Revell, 1949), that the specific cytotoxic action of the nitrogen mustards might be due to the formation of linkages between cell nuclear components.

At this stage it appeared that the methylolamides found active as tumour inhibitors were to be regarded essentially as " mustard " analogues rather than as acting like urethane, and this conception appeared to be substantiated by a more detailed study of the biological action of trimethylolmelamine. Moreover, it seemed probable that cross-linkage was in some way involved in the mechanism of action. Certain results, however, made it clear that the capacity to function as a simple cross-linking agent was not alone sufficient as a criterion of potential tumour inhibitory activity. Meanwhile the results obtained with trimethylolmelamine had already directed our attention to other chemical types having similar technological applications, namely, bis-epoxides and certain compounds containing two or three ethylenimine residues (Rose, Hendry, and Walpole, 1950), and with the discovery of tumour inhibitory activity among these substances a further chemical condition for biological activity became apparent. The results obtained with these alternative types, however, form the subject of a later communication in this series. The present paper is concerned with the initial group of substances capable of reacting with amino groups and with the methylolamides which immediately followed them.

Finally, in addition to the many compounds mentioned above, there were also included in these investigations several substances selected in connexion with another hypothesis relating to the action of urethane. Although it has never been demonstrated *in vitro*, the possibility exists that urethane, like the related isocyanate ion, might, *in vivo*, give a trimeric condensation product of the 1 : 3 : 5-triazine type or, more specifically, triethyl cyanurate. This compound, being tri-functional, might then produce cross-linking effects. It was therefore synthesized and examined together with the trimethyl ester and the corresponding acid halide, cyanuric chloride.

METHODS

As a preliminary to the examination of compounds for tumour inhibitory activity toxicity tests were carried out in mice and rats. The compounds were prepared for injection by dissolving them in physiological saline solution or in arachis oil, or in some instances in propylene glycol. Substances of limited solubility in these media were suspended by milling in arachis oil or in an aqueous solution of Dispersol OG (1.5 per cent) and Dispersol LN (0.05 per cent). They were then given to mice, usually by intraperitoneal injection, in single doses of 1,000, 500, 250, 125, and 50 mg. per kg., four mice being dosed at each level. The animals were inspected from time to time during the first half hour or so for the more readily observable signs of acute pharmacological action, such as hypnosis or convulsions, and deaths were recorded up to seven days. With the more toxic compounds the range of doses was extended downward until at least two of the four mice at any level survived.

On the basis of the results obtained in this acute test in mice, doses were chosen for repeated administration to rats. Rats were dosed once daily for five successive days in groups of three at each level. The animals were weighed each day before injection, and the doses given adjusted in proportion to the body weight. Daily weighing and recording of deaths was continued up to the tenth day. The doses employed in testing for inhibitory activity against the Walker carcinoma were based upon the results of these experiments in rats, the trends in body weight as well as deaths among the treated animals being taken into account; it was also borne in mind that tumour-bearing rats are rather more susceptible to the toxic action of many substances than are healthy animals. In the inhibitory tests dosing was usually started on the day after tumour implantation and continued daily, Sundays excepted, till the tenth to twelfth day. We aimed at giving the largest daily dose of each compound possible without any of the rats dying by the fourteenth day, and the toxicity tests described above usually sufficed for the estimation of this dose. Sometimes the condition of the treated animals made it desirable to reduce the level of dosage during the course of an experiment, at other times it was found possible to raise it; but a more precise determination of maximum tolerated doses would have required larger samples of the compounds than were usually available and a greater expenditure of time, labour, and animals than seemed expedient.

Our routine method of test for inhibitory activity against the Walker tumour, and some considerations involved in evaluating the results, have been dealt with in detail elsewhere (Walpole, 1951). Briefly the method was as follows: Single subcutaneous implants of the tumour were made by trochar and cannula in albino rats of a heterozygous stock, which were then separated into groups of 10 to 15 animals. One group was kept untreated as control and the others dosed as described above with the compounds under test. On the fourteenth or fifteenth day (the day of implantation being taken as day 0) all the surviving animals were again weighed; they were then killed and the tumours dissected out and weighed. The percentage increase in gross weight ($\triangle W$) of the survivors in each group and the percentage inhibition of tumour growth (I) in each of the treated groups were calculated. In each group I was calculated according to the formula:

$$I = \frac{M_{50}\ \text{controls} - M_{50}\ \text{treated}}{M_{50}\ \text{controls}} \times 100$$

where M_{50} was the mean weight of the n largest tumours in any group of $2n$.

RESULTS

In Table I are listed a number of "amine reactants" of various chemical types, most of which were tested before the discovery of activity in trimethylolmelamine.

TABLE I

Substances capable of reacting with amino groups examined for their effect upon the growth of the Walker tumour in rats, with the total dose in mg. per 100 g. given during the first 10–12 days after implantation of the tumour. With the exception of the three compounds in Table II, none of these produced a percentage inhibition of tumour growth (*I*) greater than 50. The effect on overall weight gain was variable. Dosage intraperitoneal unless otherwise indicated

Serial No.	Number	Compound		Total dose (mg./100g.)
		Name	Formula	
		Cyanates and isocyanates, etc.		
1	5648	Potassium cyanate	KCNO	500 *p.o.*
2	5648a	Sodium cyanate	NaCNO	80 *i.v.*
3	5475	Methyl isothiocyanate	CH_3NCS	15
4	5476	*Iso*propyl isothiocyanate	$(CH_3)_2CH.NCS$	80
5	5482	Phenylisocyanate bisulphite complex	$C_6H_5NH.CO.SO_3Na$	60
6	7957	Hexamethylenediisocyanate	$NCO.(CH_2)_6NCO$	10.5
7	5481	1 : 6-Hexamethylenediiso-cyanate bisulphite complex	$NaSO_3CO.NH(CH_2)_6NH.CO.SO_3Na$	75
8	5480	Nitrourea	$NO_2NH.CO.NH_2$	550 *p.o.*
		Ethynyl compounds		
9	6526	Phenylpropiolic acid	$C_6H_5C\equiv C.CO_2H$	120
10	6532	Ethyl *o*-chlorophenylpropiolate	$2\text{-}Cl.C_6H_4C\equiv C.CO_2C_2H_5$	120
11	6534	Tetrolic acid	$CH_3C\equiv C\ CO_2H$	60
12	6535	Ethyl tetrolate	$CH_3C\equiv C.CO_2C_2H_5$	275
		Nitriles, cyanamides, and dicyandiamides		
13	6005	Carbethoxy cyanamide (K salt)	$C_2H_5O.CO.NK.CN$	1,100
14	6184	Diethylcyanamide	$(C_2H_5)_2N.CN$	60
15	6146	Methylphenylcyanamide	$C_6H_5N(CH_3)CN$	164
16	5103	*p*-Chlorophenylcyanamide	$4\text{-}Cl.C_6H_4NH.CN$	110
17	6145	*p*-Bromophenylcyanamide	$4\text{-}Br.C_6H_4NH.CN$	55
18	6100	*ω-Iso*propyldicyandiamide	$(CH_3)_2CH.NH.C(:NH).NH.CN$	300
19	6336	β-Diethylaminoethyl dicyandiamide oxalate	$(C_2H_5)_2N.CH_2CH_2NH.C(:NH).NH.CN,\frac{1}{2}(COOH)_2$	275
20	5046	ω-(*p*-Chlorophenyl)-dicyandiamide	$4\text{-}Cl.C_6H_4NH.C(:NH)NH.CN$	220
21	6141	ω-(*o*-Chlorophenyl)-dicyandiamide	$2\text{-}Cl.C_6H_4NH.C(:NH)NH.CN$	275
22	6142	ω-(*p*-Chlorophenyl)-ω'-methyl-dicyandiamide	$4\text{-}Cl.C_6H_4NH.C(:NCH_3)NH.CN$	55
23	6829	ω-(*p*-Chlorophenylazo)-dicyandiamide (Na salt)	$4\text{-}Cl.C_6H_4N=N.NH.C(:NH)NH.CN$	162.5
24	7348	β-Diethylaminopropionitrile hydrochloride	$(C_2H_5)_2N.CH_2CH_2CN,HCl$	225

TABLE I (*continued*)

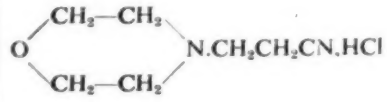
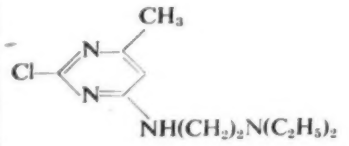
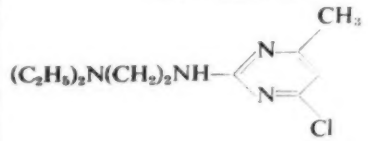
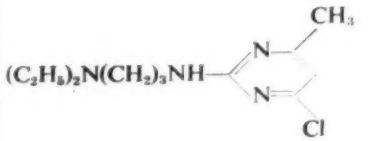
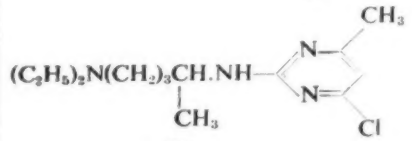
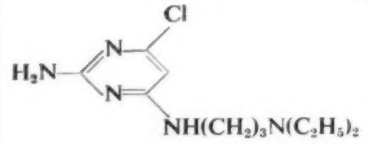
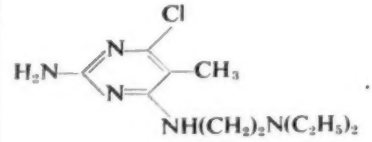
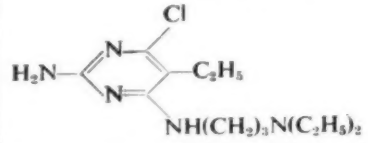
Serial No.	Number	Compound		Total dose (mg./100g.)
		Name	Formula	
		Nitriles, cyanamides and dicyandiamides (contd.)		
25	7349	β-Piperidinopropionitrile hydrochloride	$(CH_2)_5>N.CH_2CH_2CN,HCl$	22.5
26	7351	β-Hexamethyleniminopropionitrile hydrochloride	$(CH_2)_6>N.CH_2CH_2CN,HCl$	22.5
27	7350	β-Morpholinopropionitrile hydrochloride		600
		Halogenopyrimidines		
28	7744	2-Chloro-4-β-diethylamino-ethylamino-6-methyl-pyrimidine		40
29	7745	4-Chloro-2-β-diethylamino-ethylamino-6-methyl-pyrimidine		40
30	7746	4-Chloro-2-γ-diethylamino-propylamino-6-methyl-pyrimidine		40
31	7743	4-Chloro-2-`-diethylamino-α-methylbutylamino-6-methylpyrimidine		40
32	4552	2-Amino-4-chloro-6-γ-diethylaminopropylamino-pyrimidine		67.5
33	4718	2-Amino-4-chloro-6-β-diethylaminoethylamino-5-methylpyrimidine		32
34	4442	2-Amino-4-chloro-6-γ-diethylaminopropylamino-5-ethylpyrimidine		60

TABLE I (*continued*)

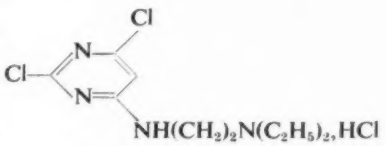
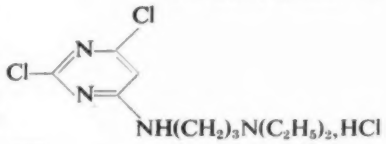
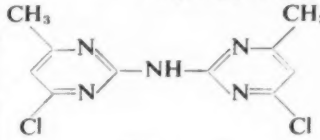
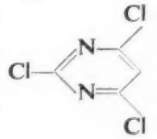
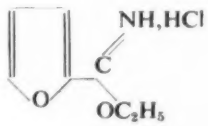
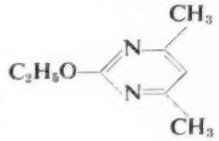
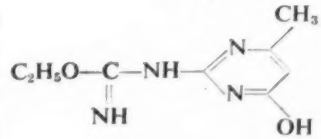
Serial No.	Number	Compound		Total dose (mg./100 g.)
		Name	Formula	
		Halogenopyrimidines (contd.)		
35	8179	2 : 4-Dichloro-6-β-diethylaminoethylamino-pyrimidine hydrochloride		40
36	8180	2 : 4-Dichloro-6-γ-diethylaminopropylamino-pyrimidine hydrochloride		45
37	8085	4 : 4′-Dichloro-6 : 6′-dimethyl-2 : 2′-dipyrimidylamine		60
38	8328	2 : 4 : 6-Trichloropyrimidine		13.5
		Esters and O-alkyl derivatives		
39	8285	N-*Iso*propyl-O-methyl*iso*urea	$(CH_3)_2CH.NH.C(:NH)OCH_3$	140
40	8284	N-*Iso*propyl-N-methyl-O-methyl*iso*urea	$(CH_3)_2CH.N(CH_3)C(:NH)OCH_3$	160
41	8283	N : N-Di-*n*-butyl-O-methyl*iso*urea	$(C_4H_9)_2N.C(:NH)OCH_3$	8
42	8282	N-*p*-Chlorphenyl-O-methyl*iso*urea	$4\text{-}Cl.C_6H_4N:C(NH_2)OCH_3$	87.5
43	6671	α-Furoiminoethyl ether hydrochloride		90
44	6065	4 : 6-Dimethyl-2-ethoxy-pyrimidine		200
45	5097	2-Carbiminoethoxyamino-4-methyl-6-hydroxypyrimidine		300
46	5142	Ethyl *p*-chlorophenyl-guanidinoformate	$4\text{-}Cl.C_6H_4HN.C(:NH)NH.CO_2C_2H_5$	4

TABLE I (*continued*)

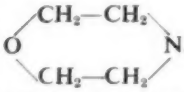
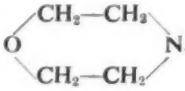
Serial No.	Number	Compound		Total dose (mg./100 g.)
		Name	Formula	
		Esters and O-alkyl derivatives (contd.)		
47	6010	Ethyl *p*-chlorophenylallophanate	4-Cl.C_6H_4NH.CO.NH.$CO_2C_2H_5$	110
48	7340	Methyl β-diethylaminopropionate hydrochloride	$(C_2H_5)_2$N.$CH_2CH_2CO_2CH_3$,HCl	700
49	7358	Methyl β-hexamethyleniminoα-methylpropionate hydrochloride	$(CH_2)_6$>N.$CH_2CH(CH_3)CO_2CH_3$,HCl	450
50	7342	Methyl β-piperidinopropionate hydrochloride	$(CH_2)_5$>N.$CH_2CH_2CO_2CH_3$,HCl	275
51	7343	Methyl β-morpholinopropionate hydrochloride		800
52	7344	Methyl β-hexamethyleniminopropionate hydrochloride	$(CH_2)_6$>N.$CH_2CH_2CO_2CH_3$,HCl	800
53	7356	Methyl α-methyl-β-piperidinopropionate hydrochloride	$(CH_2)_5$>N.$CH_2CH(CH_3)CO_2CH_3$,HCl	690
54	7357	Methyl α-methyl-β-morpholinopropionate hydrochloride		660
		Thiols and S-alkylthiols		
55	5491	Ethylthiocarbamate (thiourethane)	$NH_2CO.SC_2H_5$	75
56	5102	N-*p*-Chlorophenyl-S-methyl-*iso*thiourea sulphate	4-Cl.C_6H_4NH.C(: NH)SCH_3,$\frac{1}{2}H_2SO_4$	110
57	6349	N-Phenyl-N′-asym.dimethylguanylthiourea	C_6H_5NH.CS.NH.C(: NH)N$(CH_3)_2$	100 *s.c.*
58	6350	N-(*p*-Chlorophenylthiocarbamyl)-S-methyl*iso*thiourea	4-Cl.C_6H_4NH.CS.NH.C(: NH)SCH_3	100 *s.c.*
59	5385	N-*p*-Chlorophenyl-N′-(N″:N″-dimethylguanyl)-thiourea	4-Cl.C_6H_4NH.CS.NH.C(:NH)N$(CH_3)_2$	50
60	5403	N-*p*-Chlorophenyl-N′-(N″:N″-dimethylguanyl)-S-ethyl*iso*thiourea hydrobromide	4-Cl.C_6H_4NH.C(SC_2H_5) : N.C(:NH)N$(CH_3)_2$,HBr	50 *s.c.*
61	6143	N-*Iso*propyl-N′-cyano-S-methyl*iso*thiourea	$(CH_3)_2$CH.N:C(SCH_3)NH.CN	110
62	6414	N-Cyano-N′-phenyl-S-methyl*iso*thiourea	C_6H_5N:C(SCH_3)NH.CN	200
63	6144	N-(*p*-Chlorophenyl)-N′-cyano-S-methyl *iso*thiourea	4-Cl.C_6H_4N:C(SCH_3)NH.CN	350
		Bis- or polyfunctional esters and carbamates		
64	5606	Carbethoxyurethane	$NH(CO.OC_2H_5)_2$	550 *p.o.*
65	5609	Tricarbethoxyamine	$N(CO.OC_2H_5)_3$	1,050 *p.o.*
66	5317	Dicarbethoxyhydrazine	$C_2H_5O.CO.NH.NH.COOC_2H_5$	60 *p.o.*

TABLE I (*continued*)

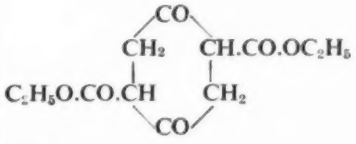
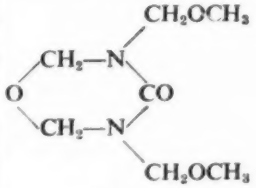
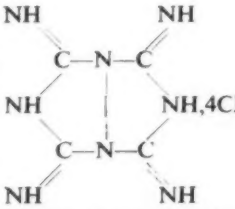
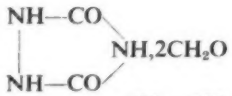
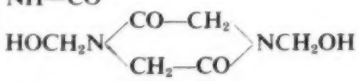
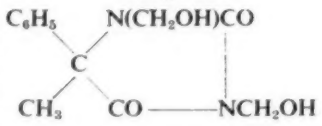
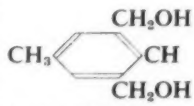
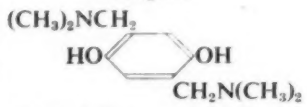
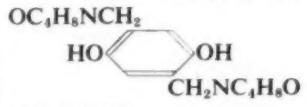
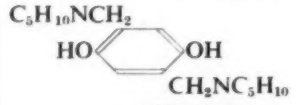
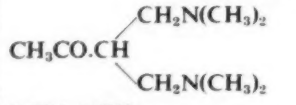
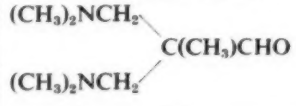
Serial No.	Number	Compound		Total dose (mg./100g.)
		Name	Formula	
		Bis- or polyfunctional esters and carbamates (contd.)		
67	5818	Dicarbethoxyethylenediamine	$C_2H_5O.CO.NH.CH_2CH_2NH.CO.OC_2H_5$	550
68	6107	Dicarbethoxypentamethylene-diamine	$C_2H_5O.CO.NH(CH_2)_5NH.CO.OC_2H_5$	200 *p.o.*
69	5819	Dicarbethoxyhexamethylene-diamine	$C_2H_5O.CO.NH(CH_2)_6NH.CO.OC_2H_5$	100
70	5993	Dicarbethoxydecamethylene-diamine	$C_2H_5O.CO.NH(CH_2)_{10}NH.CO.OC_2H_5$	550
71	5765	Ethyleneglycol bis-carbamate	$NH_2CO.OCH_2CH_2OCO.NH_2$	800
72	5766	Propyleneglycol bis-carbamate	$NH_2CO.O(CH_2)_3OCO.NH_2$	500
73	5767	Butyleneglycol bis-carbamate	$NH_2CO.O(CH_2)_4OCO.NH_2$	225
74	5814	Hexamethyleneglycol bis-carbamate	$NH_2CO.O(CH_2)_6OCO.NH_2$	350
75	5815	Decamethyleneglycol bis-carbamate	$NH_2CO.O(CH_2)_{10}OCO.NH_2$	300
76	5816	Ethyleneglycol-bis-diethyl-carbamate	$(C_2H_5)_2NCO.OCH_2CH_2OCO.N(C_2H_5)_2$	500
77	5817	Propyleneglycol bis-diethyl-carbamate	$(C_2H_5)NCO.O(CH_2)_3OCO.N(C_2H_5)_2$	200
78	7207	2:5-Dicarbethoxy*cyclo*hexane-1:4-dione		300
79	4955	N:N′-Bis-(4:6-dimethoxy-2-pyrimidyl)urea		25
		Miscellaneous methylolamides		
80	7927	Methylolurea	$NH_2.CO.NH.CH_2OH$	200
81	5429	Dimethylolurea	$HOCH_2NH.CO.NH.CH_2OH$	500
82	5430	Dimethylolurea dimethyl ether	$CH_3OCH_2NH.CO.NH.CH_2OCH_3$	300
83	9499	Dimethylolbisurea	$HOCH_2(HN.CO.NH)_2CH_2OH$	200
84	7926	Dimethylol biuret	$HOCH_2NH.CO.NH.CO.NH.CH_2OH$	250
85	3357	Bis(methoxymethyl)uron		1,050

TABLE I (*continued*)

Serial No.	Number	Compound		Total dose (mg./100g.)
		Name	Formula	
		Miscellaneous methylolamides (contd.)		
86	8376	Tetramethylolguanazo-guanazole		350
87	7925	Dimethyloladipamide	$HOCH_2NH.CO(CH_2)_4CO.NH.CH_2OH$	350
88	9402	Dimethyloldiphenylmalona-mide	$(C_6H_5)_2C(CO.NH.CH_2OH)_2$	400
89	7922	Dimethylolurazole		120
90	8103	Dimethyloldiketopiperazine		350
91	8375	Dimethylol-5-methyl-5-phenylhydantoin		200
		Miscellaneous methylol derivatives		
92	8397	2:6-Bis(hydroxymethyl)*p*-cresol		425
93	8031	2:5-Bis(dimethylaminomethyl)-hydroquinone		10
94	8032	2:5-Bis(morpholinomethyl) hydroquinone		375
95	8034	2:5-Bis(piperidinomethyl) hydroquinone		75
96	8036	1-Dimethylamino-2-dimethyl-aminomethyl-butan-3-one		6.5
97	8037	α:α-Bis(dimethylaminomethyl) propionaldehyde		20

The serial number, code number, name, and formula of each of these compounds is shown, but they will be referred to by the serial number only (or by name) in the sequel; the total dose in mg. per 100 g. rat given over the first 10 to 12 days after implantation of the tumour is also given. Unless otherwise indicated dosing was intraperitoneal. With most of these compounds any tumour inhibition produced could be accounted for on the basis of non-specific toxicity as indicated by their effect on the gross weight gain. The only substances which produced a percentage inhibition of tumour growth, *I*, of 50 or more were sodium cyanate, given intravenously, and di- and tri-carbethoxyamine (serial nos. 64 and 65), given orally. With these, some preferential effect upon the tumour was possibly in evidence, but even here activity was low. Details are shown in Table II.

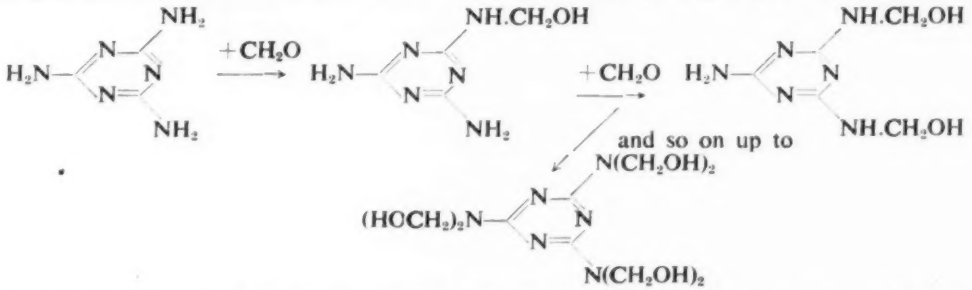
TABLE II

Substances capable of reacting with amino groups producing a percentage inhibition (*I*) of the growth of the Walker tumour in rats greater than 50 when given in the total doses shown spread over the first 10–12 days after implantation of the tumour. $\triangle W$, mean percentage increase in gross weight of the tumour-bearing rats; M_{50}, mean weight of the *n* heaviest tumours in groups of $2n$; a.s., aqueous solution; a.e., aqueous emulsion

Serial No.	Compound	Form	Total dose mg./100 g.	$\triangle W$		M_{50}		*I*
				Control	Treated	Control	Treated	
1	Sodium cyanate ..	a.s.	80 *i.v.*	30.0	−6.8	22.3	8.1	64
64	Dicarbethoxyamine (carbethoxyurethane)	a.s.	550 *p.o.*	40.2	14.8	35.9	12.4	65
65	Tricarbethoxyamine ..	a.e.	1,050 *p.o.*	37.9	6.5	33.1	14.8	55

In Table III are listed a number of "cross-linking" agents tested after the demonstration of the activity of trimethylolmelamine. None of these produced an inhibition of tumour growth greater than 45 per cent, and none appeared to have an effect which was in any sense directed preferentially towards the tumour.

Results obtained in single experiments with melamine and with the products formed by the condensation of this substance with formaldehyde in various molecular proportions are shown in Table IV, section A. These products are amorphous and difficult to characterize, and their composition is by no means established. They may be formulated provisionally as derived by the successive replacement of the hydrogen atoms in the melamine molecule by the methylol group $-CH_2OH$, thus:



In fact, however, with the exception of the hexamethylol derivative, they are probably complex mixtures of several chemical entities and very likely consist of

TABLE III

Miscellaneous " cross-linking " agents examined for their effect on the growth of the Walker tumour in rats, with the total dose in mg. per 100 g. given during the first 10–12 days after implantation of the tumour. None of these compounds produced an inhibition of tumour growth (*I*) greater than 50. The effect on gross weight gain was variable

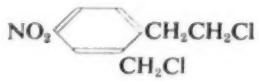
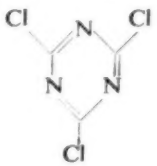
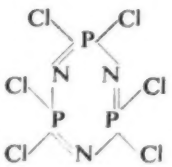
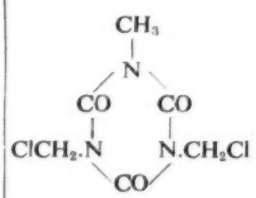
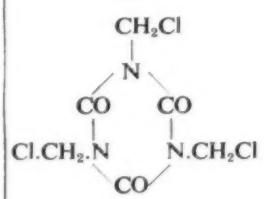
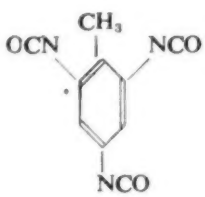
Serial No.	Number	Compound		Total dose (mg./100 g. rat i.p.)
		Name	Formula	
		Halogeno compounds		
98	9589	*p*-Xylylene dibromide		4.5
99	8361	4-β-Chloroethyl-3-chloro-methyl-nitrobenzene		25
100	8351	Cyanuric chloride		8.0
101	9418	Phosphorus chloronitride		2.25
102	8843	N: N′-bis-chloromethyl-N″-methyl isocyanuric acid		100
103	8842	N: N′: N″-Tris-chloromethyl-isocyanuric acid		65
		Isocyanates and isocyanate generators		
104	8781	Toluene tris-isocyanate		50

TABLE III (*continued*)

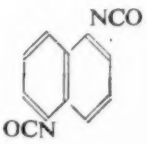
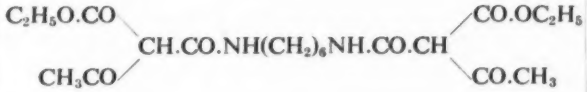
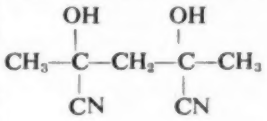
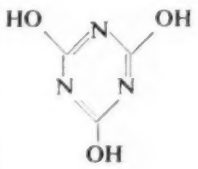
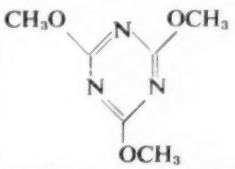
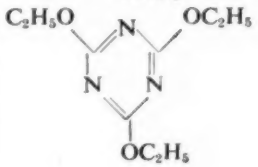
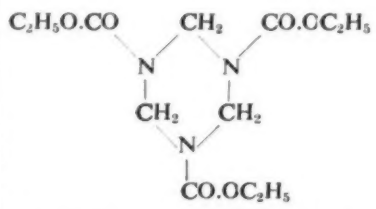
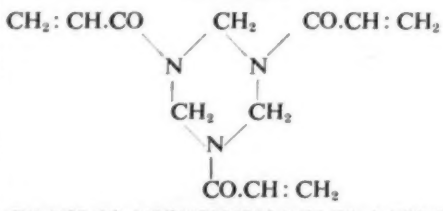
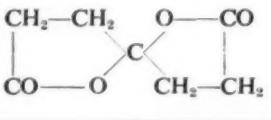
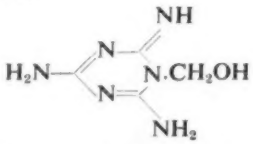
Serial No.	Number	Compound		Total dose (mg./100 g. rat i.p.)
		Name	Formula	
		Isocyanates and isocyanate generators (contd.)		
105	9861	Naphthalene-1 : 5-diisocyanate		22.5
106	9598	Hexamethylene-bis-*tert*-butylcarbamate	$(CH_3)_3CO.CO.NH(CH_2)_6NH.CO.OC(CH_3)_3$	300
107	9599	Hexamethylene diisocyanate dibenzoic anhydride	$C_6H_5CO.O.CO.NH(CH_2)_6NH.CO.O.CO.C_6H_5$	210
108	9600	N : N′-bis-(α.α-dicarbethoxy-acetyl) hexamethylene diamine	$(C_2H_5O.CO)_2CH.CO.NH(CH_2)_6NH.CO.CH(CO.OC_2H_5)_2$	275
109	9601	N : N′-bis-(α-carbethoxyaceto-acetyl)-hexamethylene diamine		325
		Aldehyde and keto derivatives		
110	9602	Glyoxal-bis-sodium bisulphite	$SO_3Na.CHOH.CHOH.SO_3Na$	325
111	9594	Acetyl acetone dicyanhydrin		14
		Acids, esters, nitriles, etc.		
112	8533	Cyanuric acid		250
113	8541	Trimethylcyanurate		100
114	8532	Triethyl cyanurate		87.5

TABLE III (*continued*)

Serial No.	Number	Compound		Total dose (mg./100 g. rat i.p.)
		Name	Formula	
		Acids, esters, nitriles, etc. (contd.)		
115	9141	N: N′: N″-Tris-carbethoxy-trimethylene triamine		145
116	9140	N: N′: N″:-Tris-acryloyl-trimethylene triamine		3.75
117	9165	Hexamethylene bis-dicyandiamide	$CN.NH.C(:NH)NH(CH_2)_6NH.C(:NH)NH.CN$	475
118	8353	Dimethyl-α: α′-azodiisobutyrate	$CH_3O.CO.C(CH_3)_2N:N.C(CH_3)_2CO.OCH_3$	50
119	8355	α: α′-Azodiisobutyronitrile	$CN.C(CH_3)_2N:N.C(CH_3)_2CN$	80
120	8354	1: 1′-Azo*cyclo*hexane carbonitrile	$(CH_2)_5>C(CN)N:N.C(CN)<(CH_2)_5$	275
121	9505	*Spiro*-anhydroketopimelic acid		175

mixtures of several of the simple substitution products formulated above. In addition, they may well contain a proportion of dimeric and/or polymeric forms containing linkages such as -NH.CH_2NH-, while the presence of a proportion of isomeric compounds of the type (IV),



(IV)

though unlikely, is not entirely excluded by the available evidence. Thus it is difficult to draw any firm conclusions as to the relationship between constitution and activity in this series. It will be seen from Table IV, however, that melamine itself proved inactive, and indeed in the experiment cited the tumour grew better in the melamine treated animals than in the controls. Condensates containing 2, 3, 4, 5, and 6 molecular proportions of formaldehyde inhibited the growth of the tumour to an extent which was clearly greater than could be accounted for by non-specific

TABLE IV

Action of some methylolmelamines and related compounds upon the growth of the Walker tumour in rats. $\triangle W$, mean percentage increase in gross weight of the tumour-bearing rats; M_{50}, mean weight of the n heaviest tumours in groups of $2n$; I, percentage inhibition of tumour growth; a.d., aqueous dispersion (suspension); a.s., aqueous solution; L, supply of compound limited, maximal tolerated dose not given

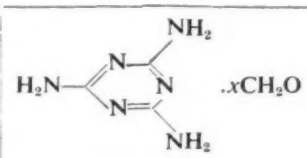
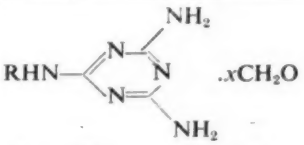
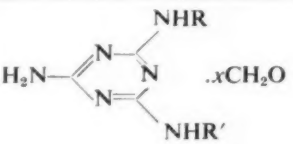
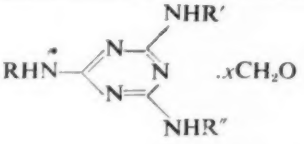
Serial No.	Number	Formula	Form	Total dose. i.p. (mg./ 100 g.)	$\triangle W$		M_{50}		I	Remarks
					Control	Treated	Control	Treated		
A	Type									
122	8455	$x = 0$	a.d.	175	29.4	21.7	19.3	23.6	−22	
123	8372	1	,,	275	44.2	20.0	30.7	17.8	42	
124	8396	2	,,	250	25.3	14.2	30.2	4.8	84	
125	7924	3	,,	450	41.8	1.2	31.9	0	100	
126	8373	4	,,	225	50.4	12.9	31.3	1.0	97	
127	8713	5	,,	237	39.1	26.4	21.8	5.6	74	
128	7923	6	,,	225	36.3	9.7	29.4	11.5	61	
B	Type									
129	8599	R = CH_3 $x = 3$	,,	265	45.6	30.6*	24.6	16.5	33	*Ascites
130	9416	C_6H_5 3	,,	250	40.0	7.8	30.4	4.4	86	
131	9205	4-Cl.C_6H_4 2	,,	112.5	33.1	−1.0	35.7	18.5	48	
132	9204	4-Cl.C_6H_4 3	,,	137.5	33.1	3.9	35.7	17.4	51	
133	9203	4-Cl.C_6H_4 4	,,	137.5	33.1	22.5	35.7	34.4	4	
134	9415	β-naphthyl 2	,,	90	32.8	24.4	40.4	35.4	8	
135	9414	β-naphthyl 3	,,	—	—	—	—	—	—	Untested *v.* Table VI
C	Type									
136	8711	R = CH_3 R′ = CH_3 $x = 3$	,,	187.5	39.1	−2.0	21.8	0.28	99	
137	8712	CH_3 CH_3 4	,,	175	39.1	15.4	21.8	1.9	91	
D	Type									
138	8575	R = CH_3 R′ = CH_3 R″ = CH_3 $x = 3$	,,	87.5	31.3	3.0	30.9	4.9	84	
139	8100	4-Cl.C_6H_4 CH_3 CH_3 2	,,	25	32.4	27.5	20.6	27.5	−33	L
140	8973	β-naphthyl CH_3 CH_3 2	,,	112.5	50.8	8.8	41.1	26.1	37	

TABLE IV (*continued*)

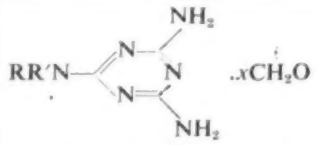
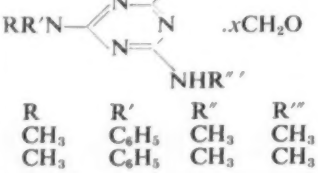
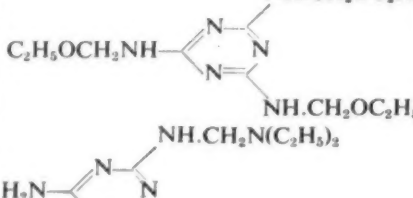
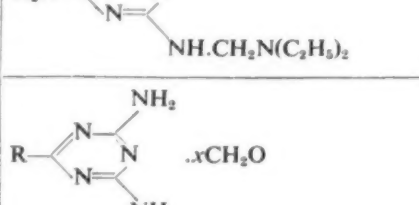
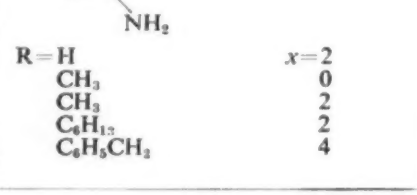
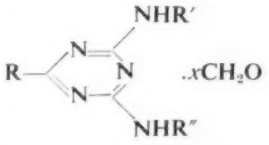
Serial No.	Number	Formula	Form	Total dose. i.p. (mg./ 100 g.)	$\triangle W$		M_{50}		I	Remarks
					Control	Treated	Control	Treated		
E	Type									
141	8578	R = CH_3 R′ = CH_3 x = 2	a.d.	106.3	43.4	16.5	12.6	9.4	25	
142	8656	CH_3 CH_3 3	,,	125	43.4	39.0	12.6	1.3	90	
143	8576	C_2H_5 C_2H_5 2	,,	192.5	31.3	26.4	30.9	20.5	34	
144	9207	CH_3 C_6H_5 2	,,	75	45.6	40.5	38.5	35.9	2	L
145	9206	CH_3 C_6H_5 4	,,	100	49.0	35.3	42.0	29.4	30	L
F	Type									
		R R′ R″ R‴ x								
146	8972	CH_3 C_6H_5 CH_3 CH_3 1	,,	125	50.8	22.2	41.1	30.8	25	L
147	9094	CH_3 C_6H_5 CH_3 CH_3 2	,,	237.5	51.0	19.4	44.3	27.8	37	
G		Miscellaneous								
148	8454		o.s.	250	40.5	19.8	25.9	5.2	80	
149	8696		a.d.	125	23.5	18.6	24.0	16.0	33	
150	8393		,,	185	39.4	23.0	30.7	24.1	22	
H	Type									
151	8456	R = H x = 2	,,	175	41.7	32.4	40.3	30.3	25	
152	8542	CH_3 0	,,	225	28.5	19.5	23.9	15.9	33	
153	8394	CH_3 2	,,	300	(25.3)	23.7	30.2	29.3	3	
154	8809	C_6H_{12} 2	,,	205	16.2	7.5	30.2	17.7	41	
155	9095	$C_6H_5CH_2$ 4	,,	140	49.6	36.4	37.4	31.7	15	L

TABLE IV (*continued*)

Serial No.	Number	Formula	Form	Total dose. i.p. (mg./ 100 g.)	△W Control	△W Treated	M_{50} Control	M_{50} Treated	I	Remarks
H *contd.*										
156	8102	R = C_6H_5—CH=CH— x = 0	a.d.	175	46.2	32.0	35.8	37.3	−4	L
157	9087	C_6H_5—CH=CH— 2	,,	230	37.8	23.8	44.7	36.3	19	
158	9088	C_6H_5—CH=CH— 3	,,	275	49.6	28.0	37.4	23.9	36	
159	9089	C_6H_5—CH=CH— 4	,,	275	49.6	24.2	37.4	29.3	22	
160	8808	α-furyl 2	,,	145	16.2	20.2	30.2	29.3	3	
161	9208	α-furyl 3	,,	125	50.0	52.5	39.9	37.8	5	L
162	8543	C_6H_5 0	,,	80	25.0	13.0	24.3	18.3	25	
163	8395	C_6H_5 2	,,	275	26.6	10.1	38.7	11.2	71	
164	8657	C_6H_5 3	,,	325	12.5	4.2	27.8	0.2	99	
165	8780	C_6H_5 4	,,	170	16.2	26.4	30.2	31.9	−6	
166	9090	4-$CH_3C_6H_4$ 2	,,	260	37.8	25.8	44.7	29.9	33	
167	9091	4-$CH_3C_6H_4$ 3	,,	275	37.8	23.5	44.7	24.0	46	
168	8974	4-$CH_3C_6H_4$ 4	,,	225	50.7	33.0	41.1	34.9	15	
169	9417	4-$CH_3O.C_6H_4$ 2	,,	270	40.0	8.4	30.4	0.8	97	
170	9092	4-$CH_3O.C_6H_4$ 3	,,	275	49.6	19.6	37.4	8.6	77	
171	9093	4-$CH_3O.C_6H_4$ 4	,,	230	49.6	25.8	37.4	11.6	69	
172	9340	4-$Cl.C_6H_4$ 2	,,	35	29.1	12.8	39.4	23.5	40	
173	9339	4-$Cl.C_6H_4$ 3	,,	39	29.1	4.9	39.4	17.7	55	
174	9338	4-$Cl.C_6H_4$ 4	,,	30	29.1	8.8	39.4	23.1	41	
175	9343	3-$Cl.C_6H_4$ 2	,,	140	42.9	22.2	34.7	21.1	39	
176	9342	3-$Cl.C_6H_4$ 3	,,	60	33.8	9.2	35.7	27.2	24	
177	9341	3-$Cl.C_6H_4$ 4	,,	60	33.8	19.3	35.7	23.2	35	
178	9346	2-$Cl.C_6H_4$ 2	,,	220	29.1	26.4	39.4	33.7	14	L
179	9345	2-$Cl.C_6H_4$ 3	,,	220	33.8	11.0	35.7	27.2	24	
180	9344	2-$Cl.C_6H_4$ 4	,,	160	33.8	18.2	35.7	31.9	10	L
181	9139	β-naphthyl 2	,,	162.5	34.6	0.9	35.4	21.2	40	
182	9086	β-naphthyl 3	,,	162.5	37.8	11.4	44.7	18.9	58	
183	9101	β-naphthyl 4	,,	275	32.4	18.5	20.6	19.3	6	
I	Type									
184	8658	R = CH_3O R′ = CH_3 R″ = CH_3 x = 2	,,	135	12.5	12.9	27.8	20.0	28	
J		Formaldehyde and Formaldehyde Generators:								
		HCHO	a.s.	20 *i.v.*	36.4	31.2	45.1	38.6	14	
		,,	,,	32 *i.v.*	17.0	15.4	39.6	30.9	22	
185	8548	$(HCHO)_n$ Paraformaldehyde	a.d.	25	36.2	27.9	29.4	27.5	6	
186	7508	$(CH_2)_6N_4$ Hexamethylene tetramine	a.s.	900	39.6	29.0	33.0	30.7	7	
187	9610	$HOCH_2SO_3Na.H_2O$ Formaldehyde sodium bisulphite monohydrate	,,	875 *i.v.*	31.6	27.8	40.4	33.6	17	L

toxicity. With monomethylolmelamine (serial no. 123) it is not clear from the figures obtained whether or not the inhibition produced (42 per cent) was due to non-specific factors.

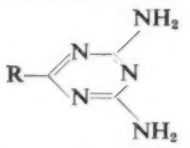
As already mentioned the doses given in these experiments were near the maximum tolerated, so that the total doses shown are inversely proportional to the toxicity of the products. It will be seen that as one proceeds from melamine to the hexamethylol derivative, increasing formaldehyde content is accompanied at first by an increase and then by a decline in tumour growth inhibitory activity. Activity reaches its maximum at or near the product analysing as trimethylolmelamine (serial no. 125). At or near this point in the series toxicity on the other hand is minimal.

The condensation between melamine and formaldehyde is reversible and the products fairly readily dissociate, liberating free formaldehyde. The question naturally arose whether the observed tumour inhibition was in fact due to the latter substance liberated *in vivo*. The total doses given of the condensates containing one to six molecular proportions of formaldehyde, expressed in terms of their formaldehyde content, were 53, 81, 187, 110, 129, and 132 mg. respectively per 100 g. rat. As far up the series as tetramethylolmelamine there is some parallelism between these figures and the observed inhibition of tumour growth, but beyond this point no such parallelism obtains. Other compounds will be found in the Tables which, in doses high in terms of readily dissociated formaldehyde, have produced no significant inhibition of tumour growth. This applies, for example, to dimethylolurea (no. 81), tetramethylolguanazoguanazole (no. 86), and dimethylolacetoguanamine (no. 153). These results all militate against the suggestion that formaldehyde liberated *in vivo* is the active agent. The results obtained with formaldehyde itself and with the simple formaldehyde generators paraformaldehyde, hexamethylene tetramine, and sodium formaldehyde bisulphite do not provide unequivocal evidence on this point. It will be seen (Table IV, J) that whereas formaldehyde given intravenously in aqueous solution and paraformaldehyde given intraperitoneally produce comparatively little inhibition of the tumour the tolerated doses are very low. While very large doses of hexamethylene tetramine and sodium formaldehyde bisulphite could be given with no greater effect on the tumour, these complexes are comparatively stable.

Results obtained with substituted methylolmelamines and with closely related derivatives of various types are shown in sections B to I inclusive of Table IV. They may be summarized in the following terms.

Activity is high in the trimethylol derivatives of alkyl and aryl substituted melamines and lower or absent in the corresponding dimethylolamines. Activity tends to be lower also in the corresponding tetramethylol derivatives.

Acyl and aroyl guanamines of the type (V)



(V)

are virtually inactive. The activity of the corresponding polymethylol derivatives depends on the nature of R as well as upon the formaldehyde content of the con-

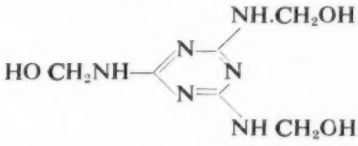
densate. Where R is hydrogen or a methyl, *n*-hexyl, benzyl, or styryl group, activity is negligible irrespective of the formaldehyde content. The same is true where R is an *α*-furyl or an *o*- or *m*-chlorophenyl group. Where R is *p*-chlorophenyl, *p*-tolyl, or *β*-naphthyl, activity is negligible in the di- and tetra-methylol derivatives, but the corresponding trimethylolamides inhibit the tumour to a rather greater extent than can be attributed to non-specific toxicity alone. Where R is phenyl or *p*-anisyl, the di- and tri-methylolamides are highly active, as is the tetramethylolamide from *p*-methoxybenzguanamine.

It was found that the water soluble trimethyl ether of trimethylolmelamine (serial no. 148) was quite active, whereas the activity of the corresponding triethyl ether (serial no. 149) was negligible.

These results will be considered further when the properties of trimethylolmelamine, one of the most active of the series, have been described in greater detail.

Trimethylolmelamine (serial no. 125)

This substance, which we formulate provisionally as (VI),



(VI)

is a white amorphous solid, sparingly soluble in water. Throughout our experiments it has been used in the form of an aqueous suspension. At first Dispersol OG (1.5 per cent) and Dispersol LN (0.05 per cent) were added to assist dispersion, but it was then found that the compound formed a fine suspension quite readily when milled with distilled water alone, and for all subsequent experiments it was made up in this way.

Tumour inhibition.—In preliminary toxicity tests in which the compound was given to mice in single intraperitoneal doses up to 1 g. per kg. none of the treated mice died up to seven days after injection. In a further test the median lethal dose in this species was found to be of the order of 1.35 g. per kg. Rats were found to tolerate five daily doses of up to *ca.* 75 mg. per 100 g. i.p. In the first test of this compound for inhibitory action on the Walker tumour, the dosage schedule per 100 g. body weight was 100 mg. on days 3, 4, and 5, 50 mg. on day 6, and 25 mg. on days 7, 10, 11, and 12, totalling 450 mg. per 100 g. i.p. Single rats died on days 12 and 14. By the fourteenth day the gross weight of the control animals had increased by 42 per cent, and the weights of the tumours in this group were 39, 34, 33, 31, 28.5, 26, 4, 0, 0, 0, 0, and 0 g. In the treated group the increase in gross weight of the survivors was only 1.2 per cent; no tumours were found although fragments of tissue of about the same size as the original implants were present in some animals.

The effect of varying the dosage and route of administration of the compound is shown in Table V. It will be seen that the degree of tumour inhibition produced depends not only on the total dosage but also upon its distribution and timing in relation to the time of implantation of the tumour. Thus a total dose of 125 mg. per 100 g. given in single doses of 25 mg. between days 1 and 6 or 1 and 7 is very

TABLE V

Effect of variations in dosage schedule and route of administration upon the inhibitory effect of trimethylolmelamine on the growth of the Walker tumour in rats. Conventions as in Table IV

Expt. No.	Route	Total dose mg./ 100 g.	Schedule of dosage	M_{50}		I	Remarks
				Control	Treated		
48/46	i.p.	450	100 on days 3, 4, and 5; 50 on day 6; 25 on days 7, 10, 11, and 12	31.9	0	100	2/12 rats dead (on days 13 and 14)
48/39	,,	250	50 on days 2, 3, and 5; 25 on days 6, 7, 8, and 9	28.0	<1	>96	2/11 rats dead (at 20 days)
48/46	,,	225	50 on days 3 and 4; 25 on days 5, 6, 7, 8, and 10	30.7	2.5	92	
	,,	120	20 on days 3 and 4; 10 on days 5, 6, 7, 8, 10, 11, 12, and 13		24.5	20	
	,,	100	100 on day 3		17.0	45	1/12 rats dead (on day 8)
48/65	,,	125	25 on days 1, 2, 5, 6, and 7	23.9	1.7	92	
48/71	,,	125	25 on days 1, 3, 4, 5, and 6	25.6	3.5	87	
48/70	,,	87.5	50 on day 1; 25 on day 2; 12.5 on day 3	25.3	2.2	91	
48/45	,,	200	50 on days 8 and 9; 25 on days 10, 12, 13, and 14	30.7	8.1		Dissected on day 15
48/65	i.v.	125	25 on day 1, 2, 5, 6, and 7	23.9	3.7	85	
48/46	p.o.	550	100 on days 3 and 4; 50 on days 5, 6, 7, 8, 10, 11, and 12	30.7	29.8	3	
48/48	,,	1,800	200 on day 3, 4, 5, 6, 7, 8, 10, 11, and 12	30.4	17.0	44	$\triangle W$ control 40.4 $\triangle W$ treated 47.2

much more effective than a total of 120 mg. given between days 3 and 13. In general the longer the time that elapses between the implantation of the tumour and the start of treatment with this compound the less marked is the effect upon tumour growth. This appears to be true also with compounds of the bis-epoxide and polyethylenimine class and will be discussed in a later paper in relation to the mode of action of compounds of all three types. Trimethylolmelamine is as active by the intravenous route as when given intraperitoneally. By mouth the compound produces much less effect, but with the enormous dose of 1.8 g. per 100 g. a 44 per cent inhibition of tumour growth was observed with no reduction in the gain in gross weight of the treated animals as compared with controls.

The compound was examined by our colleague Dr. G. T. Stewart for activity against a transmissible lymphoid leukaemia in Afb mice. Mice were inoculated with leukaemic cells and five days later dosing with the compound was begun. Three intravenous doses of 2.5 mg. per 20 g. followed by 10 of 5 mg. per 20 g., intraperitoneally, were given between the fifth and twenty-second days. This treatment caused a slight but definite retardation of the proliferation of leukaemic cells and restriction of leukaemic infiltration in the liver.

Dr. Edith Paterson, of the Christie Hospital and Holt Radium Institute, Manchester, gave trimethylolmelamine intraperitoneally to Strong A mice bearing established grafts of a spontaneous mammary tumour of that strain. The total doses given in different

groups of mice were 35, 75, 100, and 112.5 mg. per mouse in 7, 10, 10, and 9 days respectively. Tumour growth was restrained in the three latter groups, and, in the last, two permanent and three temporary regressions were seen in the seven mice treated. A dog with enlargement of the lymph nodes, liver, and spleen diagnosed as malignant lymphoma (" leukaemia ") was treated with the compound by Mr. H. B. Parry, of the Animal Health Trust. Two courses, one of five and the other of eight intravenous doses of 50 mg. per kg., were given over seven and fourteen days respectively. There was a rapid reduction in the size of the glands, the liver and spleen became no longer palpable, and the general malaise of the animal disappeared. It remained in apparent health until some 18 days later, when it contracted an acute infection (undiagnosed) and died.

Clinical trial of the compound in human malignant disease is in progress at the Christie Hospital. The results will be reported in due course.

Toxicology.—In the second experiment (48/39) recorded in Table V, the tumour weights in the treated group were estimated by palpation on the fourteenth day (mean, less than 1 g.) and the survivors killed and the tumours actually weighed on the twenty-first. By that time the weights were 15, 14.3, and 2.4 g. respectively, while in six animals only tiny nodules of tissue, necrotic in whole or part, could be found. Two rats died on day 20, one with a tumour weighing 1.7 g. and the other with no tumour. Both these animals looked anaemic shortly before death, and when the survivors in this group were killed the organs of one other were seen to be very pale. Blood taken from the heart contained 9,650 leucocytes and 1,400,000 erythrocytes per c.mm., and haemoglobin *ca.* 35 per cent. Tissues were taken *post mortem* from the treated animals in this experiment and examined histologically. Renal damage was found in all, varying in severity up to destruction of parts of the convoluted tubules. " Dead cellular " casts were often present in the lumina, and in some kidneys odd tubules were calcified. Damage was also seen around the central veins in the liver and ranged from partial destruction to full acute necrosis of the liver parenchyma in this region. These findings were later amplified by study of the effects of trimethylolmelamine in normal animals.

Experiments in rats.—No attempt will be made to detail the many experiments which have been carried out ; the essential findings are illustrated by the following. In a group of six young male rats, weighing about 100 g. at the start of experiment and dosed intraperitoneally with 50 mg. trimethylolmelamine per 100 g. on days 0–3 and 5–9 inclusive, there was marked reduction in mean body weight gain as compared with untreated controls (Fig. 1). Food intake was not recorded, and it is not known to what extent, if any, loss of appetite was responsible for this result. Individual rats died on days 16 and 17 and the remainder were killed at intervals up to the twenty-seventh day. Tissues were taken from all these animals *post mortem* for histological study. The effects of treatment upon the leucocyte and erythrocyte counts of one rat, typical of the group, are shown in Fig. 2.

A dramatic fall in the erythrocyte count some 15 to 20 days after the commencement of dosing is characteristic of the response to the compound in this species. It occurred in five of the six animals in the present experiment and was occasionally seen in rats receiving lower doses. It has not been observed in the dog even with lethal doses.

Intraperitoneal injection of the compound was followed in this experiment by a cellular reaction on the surface of the viscera—presumably a type of irritant perisplenitis, etc. The cells were large and contained abundant protoplasm. The kidneys all showed

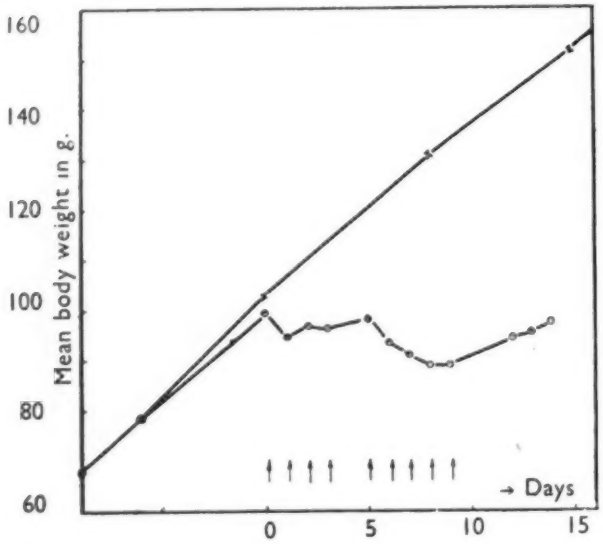


FIG. 1.—Effect of trimethylolmelamine, given intraperitoneally in aqueous suspension, upon the body weight of young male rats. ×—×: untreated controls. ⊙—⊙: rats injected at arrows with 50 mg. trimethylolmelamine per 100 g. body weight.

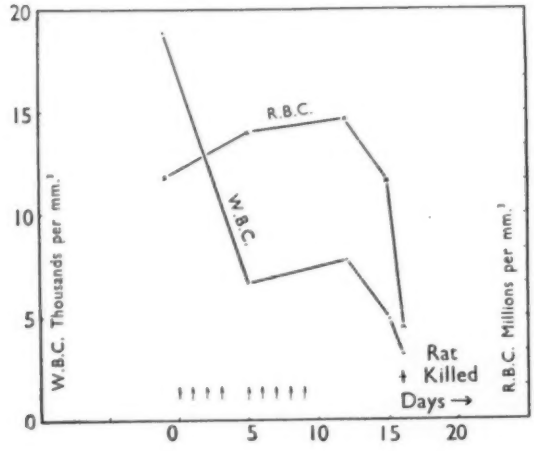


FIG. 2.—Effect of trimethylolmelamine, given intraperitoneally in aqueous suspension, upon the red and white blood cell counts of a male rat. Rat injected at arrows with 50 mg. trimethylolmelamine per 100 g. body weight.

spontaneous disease and in addition other changes certainly due to treatment. These were scarcely detectable in the rat which was killed on day 27 and were most marked in that which died on day 16. In the latter the glomeruli and first convoluted tubules were fairly normal, but the tubules of Henle and those beyond showed pathological changes. The Henle cells were highly degenerate and near necrosis. In the ducts of Bellini the tubular cells were reactive and proliferating and the lumina blocked with these and with extraneous matter which may have been the compound or a metabolite. In the rest of these rats the damage was mainly confined to the ducts of Bellini. In several animals there were minor changes in the liver (vacuolation and altered staining of the cells) in the central regions of the lobules. Throughout the series the seminiferous tubules showed advanced disorganization and spermatozoa were absent. In several instances large round cells with eosinophilic bodies in the cytoplasm were seen in the tubules, and in addition several large cells with multiple nuclei were found there. The latter were seen more consistently in the testes of rats which had received rather smaller doses of the compound and might contain anything up to 100 nuclei. Occasional cells

of this type were seen in which several of the nuclei were in division ; it is suggested that they arose from spermatocytes by repeated nuclear division without division of the cytoplasm.

Experiments in dogs.—For these studies normal dogs and bitches of the beagle breed, six to fifteen months old, were used. Trimethylolmelamine was given intravenously as a 5 per cent (w/v) aqueous suspension.

Eight doses each of 50 mg. of the compound per kg. were given over 10 days to each of 3 animals. Apart from slight depression after being dosed they appeared quite normal throughout the experiment. Treatment caused a rapid fall in the total white cell count, which reached a minimum some 20 days after the first dose and then slowly returned to normal levels. Cells of the granulocyte series were affected sooner and more markedly than the lymphocytes. There was a variable but comparatively slight downward trend in the erythrocyte count. One bitch, killed 21 days after the second of two such courses of treatment, separated by a period of 14 days, showed *post mortem* a few patches of congestion and petechial haemorrhage in the lungs and slight swelling of the glomerular endothelium in the kidney. No other pathological changes were seen in this animal.

The changes in the blood cell counts in one other of these animals (♂ 239) are shown in Fig. 3. On the 43rd day the right testicle of this animal was removed under nembutal

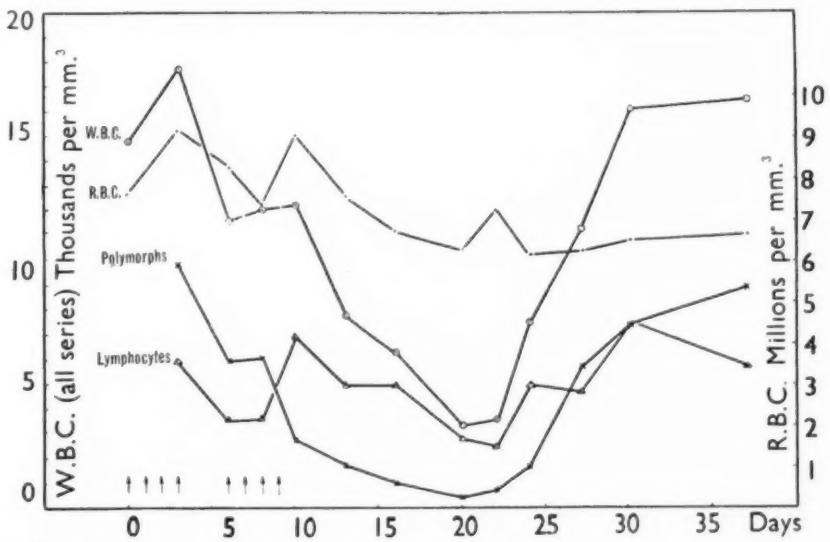


FIG. 3.—Effect of trimethylolmelamine, given intravenously in aqueous suspension, upon the blood cell counts of dog ♂ 239. Dog injected at arrows with 50 mg. trimethylolmelamine per kg.

anaesthesia. Histological examination revealed normal spermatogenesis in many of the tubules, while in others large round cells with several small dark nuclei were seen. This dog was given a second course of eight doses each of 75 mg. of the compound per kg. (days 62–65 and 68–71 inclusive). The immediate effects were slight, but a few days after the last injection the dog became listless, salivated excessively, lost appetite, and finally became comatose. It died overnight 80 days from receiving the

first (50 mg. per kg.) injection. Changes in the peripheral blood cell counts are shown in Fig. 4. The histological findings in tissues taken *post mortem* were as follows:

Lymph nodes.—There was marked central haemorrhage with local obliteration of the original structure. The peripheral ring of germinal follicles survived, but showed slight nuclear pyknosis and some depletion of lymphocytes.

Spleen.—A few lymphocytes in the Malpighian bodies showed nuclear pyknosis. The pulp was congested, and there were many haemosiderin-containing macrophages.

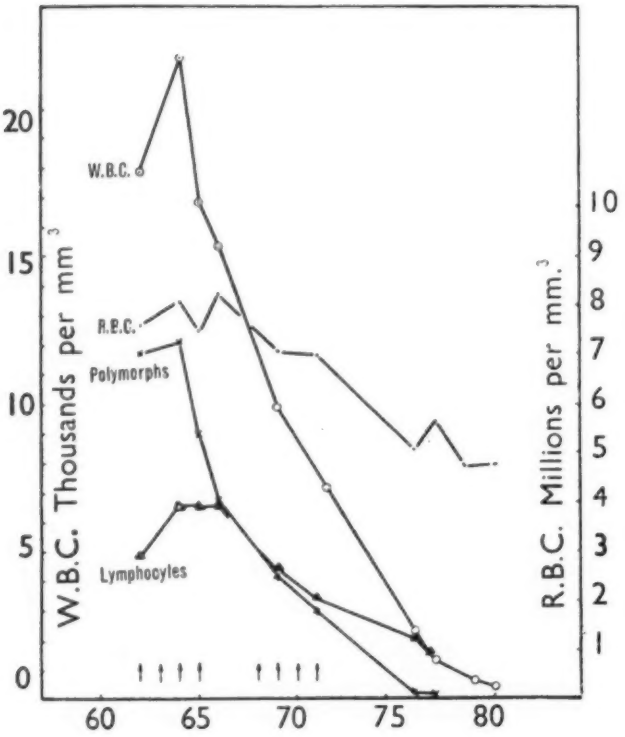


FIG. 4.—Effect of trimethylolmelamine on the blood cell counts of dog ♂ 239 continued. Dog injected at arrows with 75 mg. trimethylolmelamine per kg.

Testis.—There was no normal spermatogenesis. The lumina of the seminiferous tubules contained some large cells with numerous small nuclei, mixed with other normal cells.

Lung.—Most of the structure was lost. The outlines of some of the alveoli could still be seen, but apart from the bronchiolar cartilage almost the whole tissue was necrotic. Scattered through the section were numerous bacterial colonies, many of which had probably multiplied after the death of the animal. In considering these lung changes it should be borne in mind that the animal had been comatose some hours before death.

Liver.—There was congestion and some dilatation of the sinuses.

One other dog survived seven doses of 75 mg. of the compound per kg. and one bitch (♀ 228) succumbed to seven doses of 100 mg. per kg. (days 0–3 and 6–9 inclusive). The injection of this latter large amount was at first well tolerated although the animal vomited, defaecated, and/or urinated on several occasions shortly after being dosed and appeared somewhat depressed for an hour or two. Apart from this she appeared normal

until about four days after the last dose, when she became listless and lost appetite. By the seventeenth day she refused to eat and had become very weak ; profuse salivation was again in evidence. The blood coagulated upon dilution with Hayem's fluid and 150 mg. heparin dissolved in a little saline was given intravenously. The following day she was found moribund and was killed. White, red, and differential blood cell counts were made throughout and are plotted in Fig. 5.

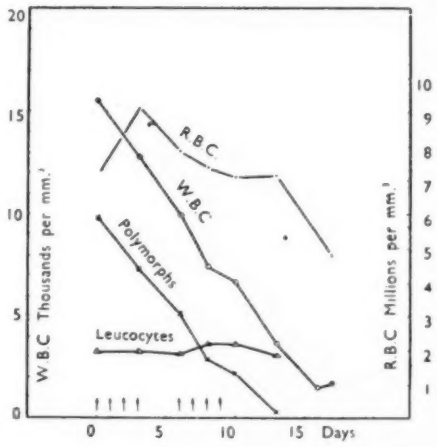


FIG. 5.—Effect of trimethylolmelamine, given intravenously in aqueous suspension, upon the blood cell counts of dog ♀ 228. Dog injected at arrows with 100 mg. trimethylolmelamine per kg.

Tissues were taken *post mortem* for histological study. In the lymph-nodes there were changes similar to those seen in dog 239, but much more severe. There was massive nuclear pyknosis and chromatorrhexis in the germinal follicles and only a few peripheral cells survived. There was depletion of lymphocytes in all areas with marked central haemorrhage. In the spleen also the damage was similar to but more severe than that seen in dog 239. Nuclear pyknosis and chromatorrhexis were very marked in the Malpighian bodies. The lungs were virtually normal. The liver sinuses in a few areas were slightly congested and some contained a granular basophilic material, possibly nuclear remains. The bone marrow taken *post mortem* from the sternum was highly abnormal. The concentration of cells was well below normal and normal mature granulocytes were absent. Such cells as were present were mainly lymphocytes, but there were some larger cells with vacuolated cytoplasm and degenerate nuclei ; these were presumably primitive cells which had undergone toxic change. There were also a few degenerate erythroblasts and normoblasts.

Cytotoxic effects (with J. M. Gates)

As it became evident that the toxic effects of trimethylolmelamine were most pronounced in proliferating tissues—tumours, bone marrow, lymphoid tissue, and the germinal epithelium—special attention was directed towards its action on dividing cells.

Mice were injected subcutaneously with the compound in aqueous suspension in single doses of 10, 20, and 40 mg. respectively per 20 g. and killed in groups of 2 to 5 animals at intervals from 2 to 72 hours later. Portions of duodenum and thymus and a splenic lymph node were taken from each animal, fixed in Bouin's fluid, imbedded in paraffin, cut at 5 μ, and stained with Heidenhain's haematoxylin.

The following nuclear abnormalities were seen in these tissues:

1. Pyknosis and fragmentation of the chromosomes, which appeared as small, intensely stained bodies scattered through the affected cells.

2. Pyknosis of whole nuclei ; these were the most frequent changes.

3. Stickiness of the chromosomes, which tended to form a central clump at metaphase and " bridges " during the anaphase separation.

4. Failure of odd chromosomes to attach themselves to the metaphase plate.

5. Very occasional tripolar mitoses.

The intensity of the damage varied with the dose and according to how long after dosing the tissues were taken. At two hours the changes were very slight and affected only a few of the dividing cells. There was a progressive increase in damage up to 24 hours when about half the dividing nuclei were involved and the damage was quite severe. In some animals the extent of the damage declined somewhat after 24 hours. It was observed that the changes in the gut (dividing cells of the crypts of Lieberkühn) were less marked than in either the thymus or lymph gland.

Nucleotoxic effects in the Walker tumour were first sought in sections of tissue taken from tumour-bearing rats killed at intervals after a single subcutaneous dose of 100 mg. of the compound per 100 g. The tissue was fixed, cut, and stained as above. Nuclear abnormalities similar to those described above were seen in these preparations.

At this stage we were greatly helped with this aspect of our investigations by Dr. P. C. Koller, of the Chester Beatty Research Institute, London, with whom we had made contact through Professor A. Haddow. Koller and his associates had made a detailed study of the changes in dividing cells exposed to *x* rays and to various chemicals and as a result had been able to define certain features of the cytotoxic action of *x* rays and of the sulphur and nitrogen mustards which seemed peculiar to those agents. These he called " specific chromosome effects " (Koller, 1949).

X rays and true " radiomimetic " agents act upon dividing cells to produce early and, if not fatal, transient changes which are not dissimilar from those obtained with cytotoxic agents of other types. Chromosome " stickiness " and its sundry manifestations, for example, fall within this category. The distinguishing feature of their action, however, is the production of permanent changes in chromosome structure. In this the primary attack upon the chromosomes seems to occur during the mitotic interphase, causing damage which becomes apparent only when the cells proceed to divide. Chromosome breakage, accompanied by the reunion of chromosome fragments to a variable extent and in various ways, leads to the appearance at anaphase, as visible manifestations of a " specific chromosome effect," of, e.g., dicentric chromosome bridges and acentric fragments. These true bridges superficially resemble those formed by the mutual adhesion of " sticky " sister chromatids but can be distinguished from them. Acentrics are small chromosome fragments lacking centromeres. Being unable for that reason to orientate themselves on the spindle they lie scattered in the equatorial region at the anaphase separation. If cell division proceeds to completion those chromosomes which are linked in a bridge may become unequally distributed between the daughter cells while some of the fragments will form accessory micronuclei. These daughter cells will have an unbalanced or deficient genetical complement and many will degenerate (v. Darlington and Koller, 1947 ; Loveless and Revell, 1949).

The sections of Walker tumour tissue from rats treated with trimethylolmelamine were examined by Dr. Koller, who confirmed the presence therein of specific chromosome changes. Twenty-four, forty-eight, and seventy-two hours after the subcutaneous injection of 100 mg. of the compound per 100 g. 84, 85, and 62 per cent respectively of the anaphases were abnormal, showing true chromosome bridges and/or numerous fragments. In addition at forty-eight and seventy-two hours many degenerating cells and cells with grossly abnormal nuclei and micronuclei were seen. At all times mitosis was rather less frequent than in control tissues, which contained some 10 per cent of abnormal anaphases, mostly showing only "sticky" bridges. One rat only was used for each of these counts. Both qualitatively and quantitatively these changes were almost indistinguishable from those produced in a parallel experiment by a dose of 0.02 mg. bis-chloroethylmethylamine per 100 g.

For the precise characterization of mitotic abnormalities squash preparations are to be preferred to sections, and in the subsequent evaluation of the changes induced in tumour tissue by our compounds we have used a technique demonstrated to us by Dr. Koller.

Animals with tumour implants of a few days' growth are treated with the compound at various dose levels. Tumour tissue taken at various times thereafter from these animals and from untreated controls is fixed in 70 per cent aqueous methanol, hydrolysed in N-HCl, and stained with Feulgen's reagent. Squash preparations are then made in 45 per cent acetic acid and restained with acetic lacmoid solution, which sharpens and intensifies the staining. After dehydration the preparations are mounted in Euparal. We examine 100 anaphases in each preparation and score the numbers showing (*a*) anaphases with true chromosome bridges, with or without fragments, (*b*) anaphases with fragments as the only visible abnormality, and (*c*) anaphases with sticky bridges. Any other cytological changes observed are recorded. Similar squash preparations are made of the bone marrow of the animals and examined both for specific chromosome changes and for general toxic effects.

Results obtained with some of the compounds mentioned above are shown in Table VI. The Walker rat carcinoma was used as the test object, and tumour tissue was sampled 24 hours after the injection of the single doses of the compounds. The corresponding figure in column A is the increase over controls in the percentage of anaphases showing abnormalities of any type produced. These figures were obtained in experiments made before the different types were separately scored, but wherever numerous the abnormalities were mainly of the specific types (*a*) and (*b*) above. The figure in column B is the increase over controls in the percentage of anaphases showing a "specific chromosome effect," i.e., abnormalities of types (*a*) and (*b*). Results of tumour growth inhibitory tests with the compounds using the Walker tumour are also shown in this Table. Within this limited selection of compounds there is evidence of a correlation between the capacity, on the one hand, to induce specific chromosome changes of the radiomimetic type, and, on the other, to inhibit the growth of the tumour. This possibility has been investigated further, more especially in connexion with the ethylenimino derivatives which form the subject of a later paper in this series, and will be dealt with in greater detail there.

Carcinogenic action

Carcinogenic activity has been demonstrated for several nitrogen mustards both of the original alkyl type (Boyland and Horning, 1949) and of the newer aryl type

TABLE VI

Comparison of cytotoxic and tumour inhibitory activity. A, percentage anaphases in excess of controls showing abnormalities of any type in tumour tissue from rats bearing the Walker tumour, 24 hr. after the single doses shown; B, percentage anaphases in excess of controls showing abnormalities of the " specific " type in tumour tissue from rats bearing the Walker tumour, 24 hr. after the doses shown; *I*, percentage inhibition of the growth of the Walker tumour in rats

Serial No.	Compound	Cytotoxic effect			Tumour inhibition	
		Dose mg./100 g. i.p.	A	B	Total dose mg./100 g. i.p.	*I*
122	Melamine	25	7		175	22
125	Trimethylolmelamine	50		47	450	100
126	Tetramethylolmelamine	20	26		225	97
174	Tetramethylol 2-p-chlorophenyl-4: 6-diaminotriazine	10	18		30	41
135	Trimethylol N-β-naphthyl melamine	100		3–9	—	—
111	Acetylacetone dicyanhydrin	4		1	14	0
107	Hexamethylene diisocyanate dibenzoic anhydride	20		0	210	34
105	Naphthalene 1: 5-diisocyanate	10		0	22.5	27

(Haddow, Horning, and Koller, 1951), and there is growing evidence of a close association of this property with tumour inhibitory action and cytotoxic activity of the nitrogen mustard (or radiomimetic) class.

Trimethylolmelamine has been on test as a carcinogen in mice and rats for periods up to two years. Intraperitoneal injection of the compound in aqueous suspension is followed by signs of local irritation and by a cellular reaction on the surface of the viscera. Subcutaneous injection produces a severe local reaction. The resulting lesion consists of a central area of coagulation necrosis surrounded by zones of oedema and inflammation. Ulceration through the skin may occur. When dosing is discontinued these lesions gradually heal.

Stock male and female albino mice of mixed ancestry were dosed subcutaneously with 1.25 mg. of the compound each per day (Monday to Friday) up to a total of 56 doses in one experiment and 70 in another. Nine months from the first injection one of the twenty-three surviving mice was found to have developed lymphoid leukaemia. No other sign of malignant disease has appeared in any other treated mouse, and this case may well have been spontaneous.

Of a group of 10 stock albino rats given a single intraperitoneal injection of 100 mg. of the compound per 100 g. one died with extensive malignant disease 17 months later. A large tumour mass was found in the lower part of the abdomen, a smaller mass adherent to the diaphragm, and numerous small nodules throughout the mesentery and lining the peritoneal cavity. Nodules were also found on the anterior wall of the thoracic cavity. This tumour was classified histologically as a highly malignant adenocarcinoma. The spleen of the animal presented a most unusual appearance. It consisted almost entirely of a mass of cells with large pale nuclei, many of which were abnormal. The remnants of the Malpighian bodies could still be recognized as shrunken atrophied structures. The cells forming the bulk of the organ bore no resemblance to those of the carcinoma.

In one rat out of a group of ten given a single subcutaneous dose of 200 mg. of the compound per 100 g. a subcutaneous spindle-celled sarcoma arose at the site of injection, some 19 to 20 months later. It is noteworthy that in this animal, killed 20 months after treatment, there was still a total absence of spermatogenesis, with cell desquamation and giant cell formation in the tubules. The persistence of testicular changes many months after the end of treatment has been observed in several animals in these experiments.

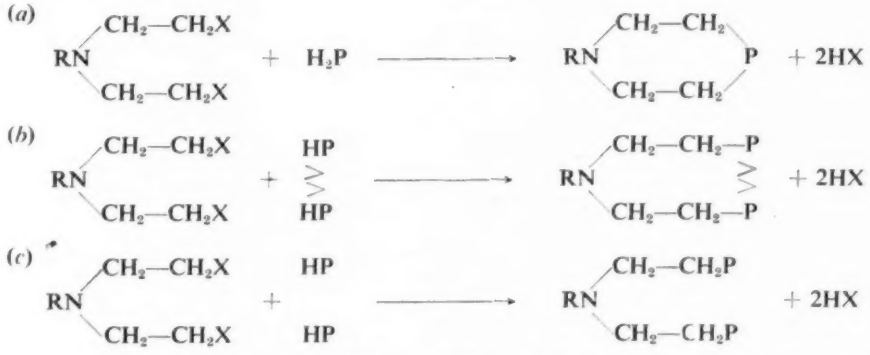
Ten rats in a third group were given two short courses, each of four daily subcutaneous doses of 50 mg. of the compound per 100 g., separated by an interval of three days. One animal in this group developed lymphoid leukaemia some 17 months after the last dose.

Eight other rats have died without tumours in these three groups. The experiment is now in the twenty-second month (December, 1950), and none of the survivors have tumours.

The commonest spontaneous tumour in our stock rats is a mammary fibroadenoma which in the later stages of its development may become malignant. Spontaneous sarcomata and carcinomata and leukaemia have also been seen from time to time so that the results described above do not constitute unequivocal evidence for carcinogenic activity. It is perhaps significant, however, that the two solid tumours in the rat experiments arose at the site of injection of the compound.

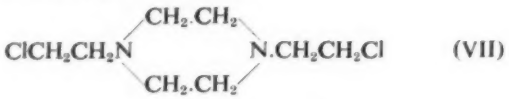
Discussion

In the preceding paragraphs we have shown that in its essential features the biological action of certain methylolmelamines closely resembles that of the nitrogen mustards. The only obvious chemical property common to these two classes is their capacity to act as alkylating agents by interaction with groupings containing active hydrogen atoms. In addition, as mentioned earlier, the characteristic biological activity of the nitrogen mustards appears to be conditional upon the presence in the molecule of at least two alkylating groups; and from our results the same appears to be true of the methylolmelamines. The interaction of a bis-functional nitrogen mustard of the type $RN(CH_2CH_2X)_2$ with cell constituents could occur in the following ways,



involving ring closure (*a*), or "cross-linkage" between two centres in the same (*b*) or separate (*c*) cellular units (P = protein).

If the alkylation of cellular constituents plays any part in the production of these biological effects it is probable that cross-linkage is involved, since with dihalogenoethylamino compounds of the type (VII)

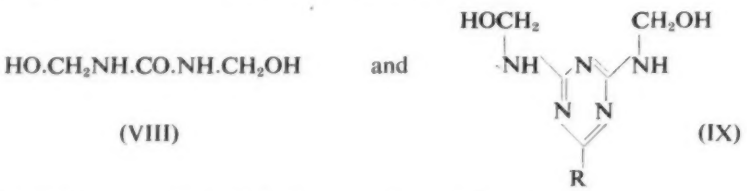


(VII)

ring closure according to scheme (*a*) above is excluded on stereochemical grounds and, as is known from the work of Burchenal and Riley (1949), compounds of this type are highly active. Assuming a symmetrical distribution of the reactive methylol groups in trimethylolmelamine our demonstration that this substance is active pointed to the same conclusion. But the main interest in this result undoubtedly lay in the fact that here for the first time cytotoxic activity of the nitrogen mustard type had been found in a structurally related but halogen-free substance which, moreover, was already in industrial use as a cross-linking agent.

At about this time, Elmore, Gulland, Jordan, and Taylor (1948), from their study of the reaction between nucleic acids, guanylic acid, and mustard gas, proposed cross-linkage by the latter substance of groups on the same or different polynucleotide chains as the possible chemical basis of its cytotoxic action, while somewhat later Goldacre, Loveless, and Ross (1949) suggested that cross-linkage might be involved in the production of chromosome abnormalities by the nitrogen mustards. Our results with trimethylolmelamine seemed to lend support to these suggestions.

On the other hand we found numerous methylolamides, which carried two prosthetic methylol groups and could therefore act as cross-linking agents, to be quite devoid of tumour inhibitory and, where tested, cytotoxic activity. These included compounds of the types (VIII) and (IX),

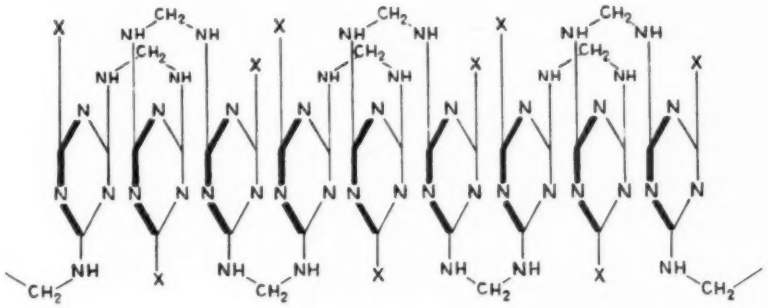


(VIII) (IX)

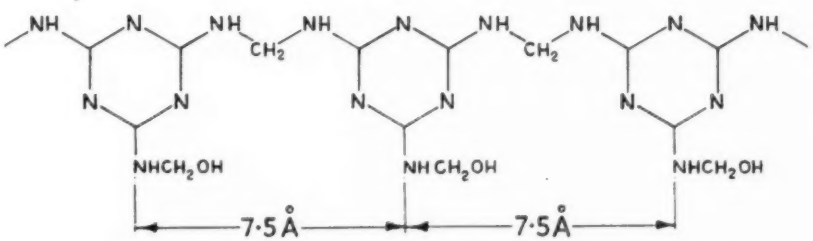
where R = H, CH_3, etc., in which the spacing of the reactive groups approximates to that of the halogen atoms in active nitrogen mustards. Certain compounds of other chemical types, and which were already known or might reasonably be expected to function as cross-linking agents, were also examined against the Walker tumour (Table III). The finding that these too were inactive further emphasized the deficiency of the hypothesis of simple cross-linkage as the basis of biological activity.

At about this time we began to examine certain epoxides and ethylenimine derivatives, and, with the discovery of nitrogen mustard-like activity in polyfunctional compounds of these two series also, a further chemical common denominator became apparent which in retrospect seemed to resolve some of the difficulties mentioned above. We were struck by the fact that the groupings in question—methylolamide, epoxide, and ethylenimine—are characterized as much by the ease

with which they polymerize as by their reactivity as alkylating agents. It seemed unlikely that this was purely fortuitous, and we began to speculate upon the possibility that the active agent in each case was not the monomeric material but a small polymer formed therefrom within the cell. Two general considerations appeared to be in harmony with this conception. The technical usage of these substances as a group is generally regarded as dependent upon the combination of the two properties mentioned above, leading to the formation of polymeric units with residual groupings capable of forming linkages between adjacent polymer chains—be they polypeptide as in wool or poly-cellobiose as in cotton fibres—with resultant modifications of structure and physico-chemical properties in the amorphous region of these fibres. It is probable, moreover, that the cellular constituents which play the fundamental role in mitosis are proteins and nucleoproteins, macromolecular structures similar in pattern to the polypeptides, and poly-cellobioses which make up the inanimate textile fibres. Unfortunately the precise nature of the polymer unit derived from trimethylolmelamine is not yet known with certainty (epoxide and ethylenimine polymers will be discussed in subsequent papers). It has been suggested (Dixon, Christopher, and Salley, 1948) that the triazine nuclei



(X)



(XI)

are arranged in it in lamellar form as represented in (X) ($X = NH.CH_2OH$); alternatively it may have an extended form in which case the most likely structure would be (XI). In either case two of the methylol groups present in each molecule of the monomer are required for the formation of the polymer chain, leaving one reactive side chain appended to each nucleus; and in either case these reactive appendages will be spaced in line at distances apart which are approximate multiples of 3.7 Å. This distance corresponds very closely to the spacing of the purine and pyrimidine residues in the nucleic acids and of the amino-acids in extended polypeptides. Such polymers would be capable of forming a comparatively stable multi-

point attachment along either protein or nucleic acid chains—such, for example, as those associated with the chromosomes and chromatids—or, in the polymers of type (X) at least, between parallel chains, to give a much more stable type of cross-linkage than that provided by unassociated molecules. If the cell components affected were indeed the chromosomes and chromatids this might well lead to the aberrations produced by the compound in dividing cells.

Assuming then that biological activity of the type under consideration depends upon the intervention of some such polymer unit it is clear that no activity would be expected from linear dimethylol compounds such as dimethylol-urea (VIII) since the derived polymers would have no residual reactive group. This would apply also to the linear dimethylolamides 82, 83, 84, 87, and 88, and further to the heterocyclic dimethylol derivatives 85, 89, 90, and 91 (Table I).

With dimethyloltriazines of the type (IX) above, in which both methylol groups would be involved in the formation of the polymer chain, the possibility of biological activity is seen to depend upon the third substituent, R, in the triazine ring. If this is inert no activity should be found ; if, on the other hand, R is a group with affinity for cellular macromolecules, becoming attached thereto either by covalent linkage or by residual valency forces or is even a group which is metabolized to a substituent having such affinity, interference with the mitotic process by the mechanism suggested above becomes possible.

In fact, no activity was found where R = H (151), Me (153), *p*-tolyl (166), *o*-chlorophenyl (178), *m*-chlorophenyl (175), *p*-chlorophenyl (172), β-naphthyl (181), α-furyl (160), styryl (157), *p*-chloroanilino (131), β-naphthylamino (134), dimethylamino (141), diethylamino (143), and methylanilino (146). Where R was phenyl (163) or *p*-anisyl (169) activity was present.

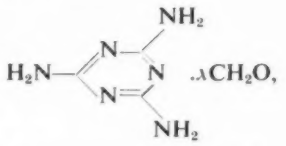
Thus the hypothesis appears to remove many of the difficulties mentioned but does not account for some few results without further assuming a metabolic change. However, the inactivity found for compounds of this type where R is a phenyl radical blocked in the *para* position by a methyl or chloro group does in some measure support the contention that metabolism of the ring is a factor concerned in the activity of the phenyl and *p*-anisyl derivatives.

The results presented and the hypothesis outlined above suggest several lines of speculation and research. The question of affinity for fibres is germane to our inquiries, and in this connexion dyestuffs and dyestuff substantivity come to mind. Substantivity in dyestuffs is usually achieved by a pattern of repeat groupings capable of binding the linear dye molecule to the fibre by residual valency forces. It would perhaps be possible to interfere with the mitotic process with such a unit or polymer chain not linked covalently with, but merely adhering to, a vital cellular component by such binding forces. Again, there is the idea, implicit in our hypothesis, that the orientation of reactive groups is a factor of importance in the process of interfering with macromolecular function. This is attained we believe with the active methylolamides by the formation of a polymer " backbone " carrying such groupings at regularly spaced intervals, but we have further suggested (Hendry, Rose, and Walpole, 1950) that a similar orientation of prosthetic groups might be brought about solely by micelle formation, perhaps, for instance, in the carcinogenic polycyclic hydrocarbons. Work in this connexion is in process and will be reported in due course.

SUMMARY

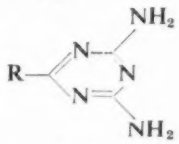
A description is given of the sequence of events leading to the discovery of tumour inhibitory activity, against the transplanted Walker carcinoma 256 in rats, in polymethylolamides prepared by condensing formaldehyde with melamine and with certain derivatives of that substance.

Compounds of the general formula



containing two or more molecular proportions of formaldehyde ($x = 2$–6) have been found active. Activity is high in the trimethylol derivatives of alkyl and aryl substituted melamines and lower or absent in the corresponding dimethylolamides.

Acyl and aroyl guanamines of the type



are inactive. When R is hydrogen, methyl, *n*-hexyl, benzyl, styryl, α-furyl, or *o*- or *m*-chlorophenyl, the corresponding polymethylol derivatives are of negligible activity irrespective of formaldehyde content. When R is *p*-chlorophenyl, *p*-tolyl, or β-naphthyl the same is true of the di- or tetra-methylol derivatives, but the corresponding trimethylolamides have some activity. When R is phenyl or *p*-anisyl the di- and tri-methylolamides are highly active, as is the tetramethylolamide from *p*-methoxybenzguanamine.

Certain polymethylolamides are employed industrially for modifying the mechanical and physico-chemical properties of textile fabrics. Their usefulness in this direction is probably associated with their poly-alkylating function and with the formation of cross-linkages between adjacent polymer fibres. Our attention had been drawn to the poly-alkylating and cross-linking potentialities of the sulphur and nitrogen mustards, and it had been suggested that the specific cytotoxic effects and tumour inhibitory action of the latter might be due to the formation of cross-linkages between cell nuclear components. It appeared that the biological properties of the mustards and of the active polymethylolamides might have a common foundation. Support for this view has been obtained from a more detailed study of the biological effects of trimethylolmelamine, taken as representative of active compounds of the latter type. The toxic action of this substance in the animal body is directed mainly against those tissues where cell division is most active—the haematopoietic and lymphoid systems, the intestinal epithelium, and the germinal epithelium of the testis —with the production of minor pathological changes in epithelia and endothelia in other sites. In addition it produces in dividing cells of the Walker tumour specific chromosome effects—chromosome fragmentation and " bridge " formation—characteristic of the action of true radiomimetic agents. Tests for carcinogenic activity have given equivocal results.

The hypothesis of simple cross-linkage as the basis of biological activity is insufficient, however, to account for all the facts. In particular many bis-functional compounds which are known, or might reasonably be expected, to act as cross-linking agents have been found to be quite inactive against the Walker tumour. We postulate as an essential step in the production of their biological effects the intermolecular condensation of the methylolamides into polymeric units, in which two methylolamide groups in each molecule are involved in the formation of the polymer chain. A third active centre is then required, which may be a methylolamide group or one with similar functional capacities, which serves to attach the unit at several points along or between protein or nucleic acid chains—such as those associated with the chromosomes—in the cell.

We are indebted to Misses S. Bentley, S. Jones, and B. Lowe for technical assistance and to Dr. Edith Paterson and Mr. H. B. Parry for permission to quote unpublished results.

References

Boyland, E., and Horning, E. S. (1949). *Brit. J. Cancer*, **3**, 118.
Burchenal, J. H., and Riley, J. B. (1949). *Cancer Research*, **9**, 553.
Darlington, C. D., and Koller, P. C. (1947). *Heredity*, **1**, 187.
Dixon, J. K., Christopher, G. L. M., and Salley, D. J. (1948). *Paper Trade J.*, **127**, 455.
Elmore, D. T., Gulland, J. M., Jordan, D. O., and Taylor, H. F. W. (1948). *Biochem. J.*, **42**, 308.
Evans, J. G. (1949). *Fibre Science*, Textile Institute, Manchester, p. 248.
Gilman, A., and Philips, F. S. (1946). *Science*, **103**, 409.
Goldacre, R. J., Loveless, A., and Ross, W. C. J. (1949). *Nature, Lond.*, **163**, 667.
Haddow, A., Kon, G. A., and Ross, W. C. J. (1948). *Nature, Lond.*, **162**, 824.
Haddow, A., Horning, E. S., and Koller, P. C. (1951). In press.
Haddow, A., and Sexton, W. A. (1946). *Nature, Lond.*, **157**, 500.
Hendry, J. A., Rose, F. L., and Walpole, A. L. (1950). *Proceedings of the Fifth International Cancer Congress*, Paris, p. 88.
Karnovsky, D. A., Burchenal, J. H., Ormsbee, R. A., Cornman, I., and Rhoads, C. P. (1947). *Approaches to Tumour Chemotherapy*, Washington, D.C., p. 293.
Koller, P. C. (1949). *Hereditas*, Suppl. Vol., 320.
Lefèvre, J. (1939). *C.R. Acad. Sci., Paris*, **208**, 301.
Loveless, A., and Revell, S. (1949). *Nature, Lond.*, **164**, 938.
Paterson, E., apThomas, I., Haddow, A., and Watkinson, J. M. (1946). *Lancet*, **1**, 677.
Rose, F. L., Hendry, J. A., and Walpole, A. L. (1950). *Nature, Lond.*, **165**, 993.
Skipper, H. E., and Bryan, C. E. (1949). *J. Nat. Cancer Inst.*, **9**, 391.
Skipper, H. E., Bryan, C. E., Riser, W. H., jun., Welty, M., and Stelzenmuller, A. (1948). *J. Nat. Cancer Inst.*, **9**, 77.
Templeman, W. G., and Sexton, W. A. (1945). *Nature, Lond.*, **156**, 630.
Walpole, A. L. (1951). *Brit. J. Pharmacol.*, **6**, 135.

Brit. J. Pharmacol. (1951), **6**, 235.

CYTOTOXIC AGENTS: II, BIS-EPOXIDES AND RELATED COMPOUNDS

BY

J. A. HENDRY, R. F. HOMER, AND F. L. ROSE

From Imperial Chemical Industries, Ltd., Research Laboratories, Hexagon House, Manchester, 9

AND

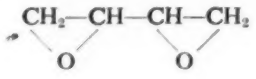
A. L. WALPOLE

From Imperial Chemical Industries, Ltd., Biological Laboratories, Wilmslow, Cheshire

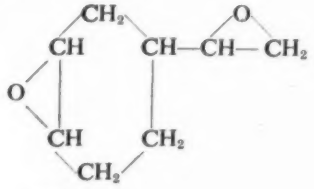
(Received February 22, 1951)

In Part I (Hendry, Rose, and Walpole, 1951) of the present series of papers, we described the discovery of "radiomimetic" activity in a series of methylolamide derivatives. Although this discovery emerged from investigations undertaken on the supposition that urethane might function as a chemically reactive grouping towards amines of cellular origin, it became apparent that the biological effects of these substances were more closely related to their use in textile technology. The "cross-linking" action, upon which their effects upon textile fibres seem to depend, might well lead to the linkage of cellular components in a manner analogous to that which has been suggested for the nitrogen mustards, and which is believed by Haddow and his co-workers to be responsible for the specific cytotoxic effects of the latter. In fact the first methylolamides which we examined were selected from a number made available to us in 1947 by our colleagues who were studying their use in the textile field. This technological interest extended to compounds of other chemical types having similar applications and similar "cross-linking" potentialities, notably certain epoxide and ethylenimine derivatives, and these by analogy were deemed worthy of biological examination. This communication concerns substances of the former type, namely, those based on the ethylene oxide ring system. Brief reference to them has been made in an earlier note (Rose, Hendry, and Walpole, 1950).

The chemistry of these substances has been intensively investigated during the past six or seven years in the laboratories of Canadian Industries Ltd., Montreal, from whom some of our earlier specimens were obtained, including the important dioxide (II) from butadiene dimer (4-vinyl *cyclo*hexene). For the butadiene dioxides (I) we are indebted to our colleague Dr. W. F. Beech.



(I) 8838 (mixed isomers) and 9137 (*meso*)



(II) 8840

The selection of compounds for examination was based upon a number of premises which were modified or added to as the work proceeded. Since our findings in the methylolamide series suggested that polyfunctionality was essential for activity, structures carrying two epoxide groups were first examined as tumour inhibitors (initially against the transplanted Walker carcinoma) and several found to be active. It was nevertheless considered desirable to check the presumed inactivity of mono-epoxides. Some 25 compounds were therefore examined in which a single epoxide radical was linked to a wide variety of groupings, including acyclic and carbocyclic structures giving substances, e.g., those with serial numbers 203, 196, 197, and 199 (Table I) related to butadiene and vinyl *cyclo*hexene dioxides respectively, and also basic groupings, as 205 and 206. In some instances, because of ease of access, the epoxide ring was introduced in the form of a derivative of glycidol ; in others the oxide was obtained, indirectly, by oxidation from the corresponding olefine. In view of the possibility of *in vivo* oxidation it was considered desirable to examine the parent hydrocarbons corresponding not only to the mono-epoxides but also to the active bis-epoxides. Some monoene mono-epoxides were also examined. In addition, examples of (mono) epoxides derived from certain terpenoid compounds (limonene, safrole) were included because of the special interest which attached to these potential epoxide precursors of natural occurrence.

The bis-epoxides as a whole were similarly diverse in character. Since the specific biological effects observed were at first believed to depend upon the capacity of each of the epoxide groups to react with groupings carrying active hydrogen ($R—\underset{\backslash O /}{CH}—CH_2 + HX \rightarrow R—\underset{|}{\underset{OH}{CH}}—CH_2—X$), such factors as the steric and inductive effects of proximal parts of the molecule were regarded as important. It was hoped that the significance of these factors and in addition that of spatial considerations, such as the distance between the epoxide groups, could be deduced from a study of the results obtained with the variety of examples of the bis-functional type examined.

In Part I we drew attention to the carcinogenic potentialities of tumour inhibitory cytotoxic agents of the radiomimetic class and described our failure unequivocally to demonstrate tumour production. We have tested two active members of the present series as carcinogens and shown that one of them readily produces malignant tumours in the mouse and the rat. The mutagenic activity of the nitrogen mustards is well known, and here, too, the association with specific cytotoxic activity seems close. The one bis-epoxide tested for such activity has produced mutation in a strain of *Penicillium*.

Methods

Preliminary toxicity tests in mice and rats were carried out on the compounds in the same way as described in the previous paper for those which formed the subject of that report (Hendry, Rose, and Walpole, 1951). The majority were oil soluble and were given in solution in arachis oil. A few were dissolved in water or suspended by milling in an aqueous solution of Dispersol OG (1.5 per cent) and Dispersol LN (0.05 per cent). Many of the epoxides are decomposed in aqueous solution, and with these substances water was as far as possible avoided as a solvent. From the results of these tests an estimate was made of the highest level at which rats bearing the Walker tumour

could be dosed daily, Sundays excepted, for the first 10 to 12 days of experiment without any of the rats dying by the fourteenth. This schedule of dosage, starting the day after implantation of the tumour, was used in the initial tests for tumour inhibitory activity.

Our routine method of test for inhibitory activity against the Walker tumour has also been described (Walpole, 1951). The percentage increase in gross weight ($\triangle W$) of the survivors in each group of rats and the percentage inhibition of tumour growth (I) in each of the treated groups was calculated, the latter according to the formula

$$I = \frac{M_{50} \text{ controls} - M_{50} \text{ treated}}{M_{50} \text{ controls}} \times 100$$

where M_{50} was the mean weight of the n largest tumours in any group of $2n$.

Results

In Table I are listed all the compounds which come under consideration. The serial and code number, name and formula of each is shown together with the total dose in mg. per 100 g. rat given over the first 10 to 12 days after implantation of the tumour. Dosing was invariably intraperitoneal. Compounds will be referred to in the sequel by their serial numbers.

It will be seen that the unsaturated hydrocarbons examined (serial nos. 188–195) produced little or no inhibition of tumour growth in maximal tolerated doses. Several of these compounds are potential precursors of the active di-epoxide, 4-vinyl *cyclo*hexene dioxide, and this result disposes of the suggestion that they might be oxidized to the latter *in vivo* to any appreciable extent. 1 : 5-Hexadiene (195) is also virtually inactive, so that it is unlikely that this is oxidized in the body to the corresponding bis-epoxide, reported active by Ross (1950).

The mono-epoxides which follow (serial nos. 196–220) are similarly inactive or of low activity ; the tumour inhibition produced was never clearly greater than could be accounted for by the general toxic action. These compounds include 4-vinyl *cyclo*hexene monoxide (196), and the fact that this compound has but little action at a very high dose level leads to a conclusion similar to those drawn above. The results with the mono-epoxides taken as a whole support the contention that the presence of only one epoxide group in a molecule is insufficient to give rise to activity.

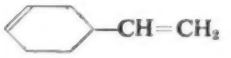
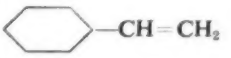
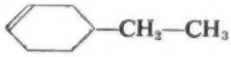
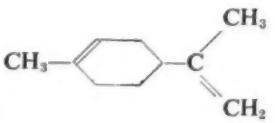
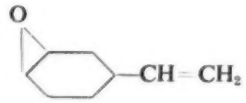
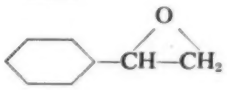
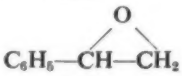
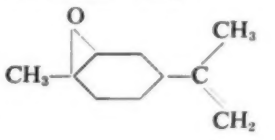
Of the bis-epoxides examined, however (serial nos. 221–240), several were found to produce very considerable inhibition of tumour growth, although even here there is a considerable variation in activity. Even with the most active, however, the margin between the lethal dose and the dose required to produce marked inhibition of tumour growth is small, and for that reason we consider it unlikely that any of this type will be found to have any therapeutic application.

Cytotoxic effects

A selection of mono- and bis-epoxides and related compounds has been examined for activity in the production of specific chromosome damage in dividing cells of the Walker tumour. Single intraperitoneal doses of the different compounds were given to tumour-bearing rats, and Feulgen-stained squash preparations were made from tumour tissue taken when the animals were killed 24 hours later. Similar preparations were made of bone marrow taken from the rats immediately after they

TABLE I

Action of some epoxides and related compounds upon the growth of the Walker tumour in rats. $\triangle W$ = mean percentage increase in gross weight of the tumour-bearing rats;

Serial No.	Code Number	Compound	
		Name	Formula
		HYDROCARBONS	
		(*a*) *Related to vinyl*cyclo*hexene*	
188	9448	4-Vinyl*cyclo*hexene*	
189	9449	Vinyl*cyclo*hexane*	
190	9510	4-Ethyl*cyclo*hexene*	
191	9254	*dl*-Limonene (dipentene)	
192	9109	Styrene	$C_6H_5—CH=CH_2$
193	9294	1-Phenylbutadiene	$C_6H_5—CH=CH—CH=CH_2$
194	9291	α-Vinylnaphthalene	α-$C_{10}H_7CH=CH_2$
		(*b*) *Related to* 1 : 3-*butadien*	
195	9135	1 : 5-Hexadiene	$CH_2=CH—CH_2—CH_2—CH=CH_2$
		MONOEPOXIDES	
		(*a*) *Related to vinyl*cyclo*hexene monoxide*	
196	9446	4-Vinyl*cyclo*hexene monoxide	
197	9515	Vinyl*cyclo*hexane oxide	
198	9447	*Cyclo*hexene oxide	
199	9110	Styrene oxide	
200	9297	*dl*-Limonene monoxide	

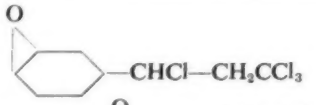
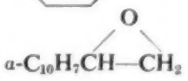
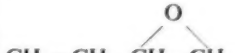
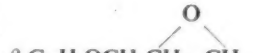
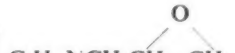
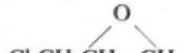
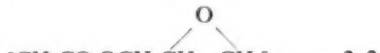
*Obtained from Canadian Industries Ltd.

TABLE I (*continued*)

M_{50} = mean weight of the n heaviest tumours in groups of $2n$; I = percentage inhibition of tumour growth; o.s. = oily solution; a.s. = aqueous solution; a.d. = aqueous dispersion

Form	Total dose (mg./100 g. i.p.)	Tumour inhibition				
		$\triangle W$		M_{50}		I
		Control	Treated	Control	Treated	
o.s.	480	48.0	36.3	42.1	38.4	9
,,	325	48.0	43.7	42.1	36.8	13
,,	300	45.3	47.6	28.5	37.0	0
,,	275	29.1	29.1	39.4	35.6	10
,,	425	38.5	35.3	35.4	28.9	18
,,	185	35.1	29.6	36.1	38.2	0
,,	325	46.2	47.1	36.9	39.0	0
,,	50	45.6	33.4	38.5	31.2	19
,,	400	48.0	20.5	42.1	26.7	37
,,	300	17.1	24.2	35.1	35.3	0
,,	170	39.8	14.0	30.3	25.7	15
,,	220	34.6	20.7	35.4	29.2	18
,,	300	36.7	23.7	37.2	23.7	36

TABLE I (*continued*)

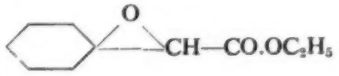
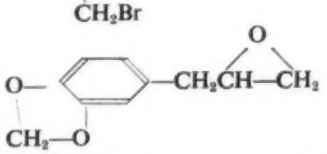
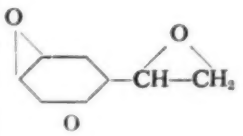
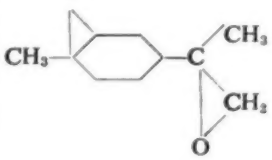
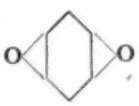
Serial No.	Code Number	Compound	
		Name	Formula
201	9560	4-(α : γ : γ : γ-tetrachloropropyl) *cyclo*hexene oxide	
202	9444	α-Vinylnaphthalene oxide	 Untested *v.* Table II
		(*b*) *Related to* 1 : 3-*butadiene monoxide*	
203	9136	1 : 3-Butadiene monoxide	
		(*c*) *Derivatives of glycidol*	
		(i) *Ethers*	
204	3928	β-Naphthylglycidyl ether	
		(ii) *Amines*	
205	9252	Diethylamino epihydrin	
206	9293	Piperidino epihydrin	
207	9621	N(2 : 3-epoxy-*n*-propyl)-*p*-chloraniline	
		(iii) *Alcohols and esters*	
208	2338	Glycidol	
209	9096	Epichlorhydrin	
210	9593	Glycidyl acetate	
211	9595	poly-Glycidyl acetate	
212	10061	Glycidyl acrylate*	
213	10062	Glycidyl methacrylate*	

*Obtained from Canadian Industries Ltd.

TABLE I (*continued*)

Form	Total dose (mg./100 g. i.p.)	Tumour inhibition				
		ΔW		M_{50}		I
		Control	Treated	Control	Treated	
o.s.	350	40.8	41.4	32.8	32.1	2
,,	135	55.1	19.5	36.5	20.8	43
,,	300	36.7	18.6	37.2	26.0	30
,,	60	45.6	26.3	38.5	29.0	25
a.s.	80	35.1	24.9	36.1	34.7	4
o.s.	275	34.4	16.6	39.7	30.6	23
a.s.	140	30.7	27.5	31.7	37.4	0
o.s.	42	33.1	27.5	35.7	26.0	27
,,	70	17.2	27.2	35.1	35.9	0
,,	325	29.9	22.2	43.1	33.8	22
,,	3.5	26.4	26.4	33.6	33.6	0
,,	150	32.6	12.5	42.6	26.0	39

TABLE I (*continued*)

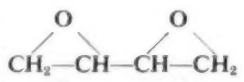
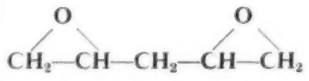
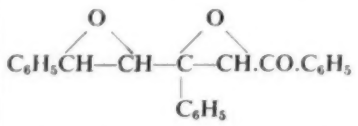
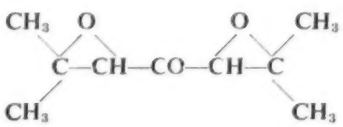
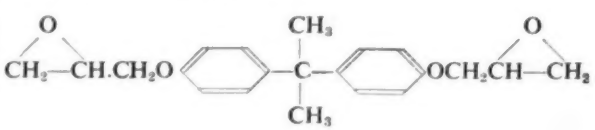
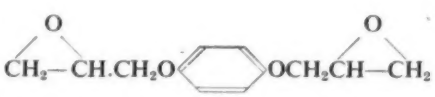
Serial No.	Code Number	Compound		
		Name	Formula	
214	10209	Glycidyl oleate	$C_8H_{17}CH{=}CH.C_7H_{14}CO.OCH_2CH(O)CH_2$	Untested. See Table II
215	9893	Glycidyl stearate	$C_{17}H_{35}CO.OCH_2CH(O)CH_2$	
		(*d*) *Miscellaneous*		
216	9366	α: β-Epoxymesityl oxide	$(CH_3)_2C(O)CH.CO.CH_3$	
217	9512	2-Carbethoxy-1-oxa*spiro*-2 : 5-octane		
218	9513	Sodium phenylglycidate	$C_6H_5{-}CH(O){-}CH.CO_2Na$	
219	9615	α-Phenyl-α-bromomethyl ethylene oxide	$C_6H_5{-}C(CH_2Br)(O){-}CH_2$	
220	9643	Safrole oxide		
		DI-EPOXIDES		
		(*a*) *Related to vinyl*cyclo-*hexene dioxide*		
221	8840	4-Vinyl*cyclo*hexene dioxide*		
222	9253	*dl*-Limonene dioxide		
223	10058	1 : 4-*cyclo*hexadiene dioxide*		

*Obtained from Canadian Industries Ltd.

TABLE I (*continued*)

Form	Total dose (mg./100 g. i.p.)	Tumour inhibition				
		$\triangle W$		M_{50}		I
		Control	Treated	Control	Treated	
o.s.	300	36.7	31.8	37.2	27.5	26
,,	300	45.3	42.7	28.5	33.1	0
a.s.	300	45.6	45.2	45.5	48.0	0
o.s.	16	27.4	14.4	26.0	25.5	2
,,	150	17.3	5.7	29.8	25.7	14
,,	225	40.6	13.0	39.5	2.5	94
,,	265	51.1	35.0	44.2	34.4	22
a.s.	150	37.3	8.0	44.6	25.3	43

TABLE I (*continued*)

Serial No.	Code Number	Compound	
		Name	Formula
		(*b*) *Related to* 1 : 3-*butadiene dioxide*	
224	8838	1 : 3-Butadiene dioxide (mixed isomers)	
225	9137	Meso-1 : 3-butadiene dioxide	
226	10059	1 : 4-Pentadiene dioxide*	
227	9561	1 : 3-Diphenyl-4-benzoyl-1 : 3-butadiene dioxide	
228	9445	Phorone dioxide	
		(*c*) *Derivatives of glycidol*	
		(i) *Ethers*	
229	8839	Diallyl ether dioxide	
230	9367	Ethylene glycol diglycidyl ether	
231	10317	Diethylene glycol diglycidyl ether*	
232	10060	Butane-1 : 4-diol diglycidyl ether*	
233	9514	Diphenylolpropane diglycidyl ether	
234	9364	Hydroquinone diglycidyl ether	

*Obtained from Canadian Industries Ltd.

TABLE I (*continued*)

Form	Total dose (mg./100 g. i.p.)	Tumour inhibition				
		$\triangle W$		M_{50}		I
		Control	Treated	Control	Treated	
o.s.	16	32.4	5.9	20.6	0.6	97
,,	5	50.1	19.2	39.8	18.0	55
,,	250	26.4	16.9	33.6	23.8	29
a.d.	200	40.8	39.2	32.8	31.5	4
o.s.	80	43.3	49.3	36.0	39.9	0
,,	20	32.7	6.7	40.4	21.5	47
,,	50	48.7	5.2	45.05	16.7	63 at 15 days
,,	150	26.0	14.7	31.2	4.9	84
,,	120	31.8	2.2	46.7	12.2	74
,,	400	43.3	32.7	36.0	37.2	0
a.d.	80	26.6	−0.3	39.0	23.0	41

TABLE I (*continued*)

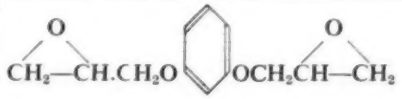
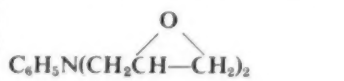
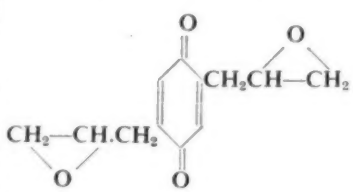
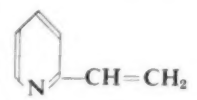
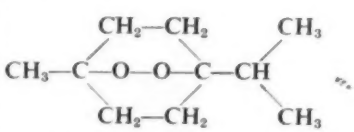
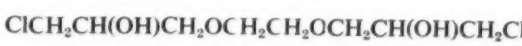
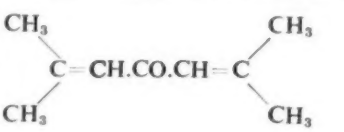
Serial No.	Code Number	Compound	
		Name	Formula
235	9511	Resorcinol diglycidyl ether	
		(ii) *Amines*	
236	9295	N: N-di-(2′: 3′-epoxy-*n*-propyl)-*p*-phenetidine	
237	9559	N: N-di-(2′: 3′-epoxy-*n*-propyl)*p*-anisidine	
238	9588	N: N-di-(2′: 3′-epoxy-*n*-propyl)-*p*-toluidine	
239	9587	N: N-di-(2′: 3′-epoxy-n-propyl)-aniline	
		(*d*) *Miscellaneous di-epoxides*	
240	9368	2: 5-di-(2′: 3′-epoxy-*n*-propyl)-benzoquinone	
		MISCELLANEOUS COMPOUNDS	
241	9296	N: N-di-(3′-chloro-2′-hydroxy-*n*-propyl)-*p*-phenetidine	
242	9365	2-Vinylpyridine	
243	9373	Ascaridole (oil of wormseed)	
244	9622	Ethyleneglycol-di-(3′-chloro-2′-hydroxy-*n*-propyl)ether	
245	8841	Phorone	

TABLE I (*continued*)

Form	Total dose (mg./100 g. i.p.)	Tumour inhibition				
		$\triangle W$		M_{50}		I
		Control	Treated	Control	Treated	
o.s.	35	45.6	17.1	45.5	33.1	27
,,	350	50.0	6.2	38.2	2.3	94
,,	170	34.4	−4.1	39.7	4.5	86
	190	24.5	10.0	32.4	28.6	12
,,	190	24.5	7.7	32.4	21.6	33
,,	275	26.6	25.2	39.0	36.8	6
a.d.	200	35.1	29.0	36.1	31.5	13
o.s.	75	26.6	28.8	39.0	33.3	15
,,	20	17.3	22.2	29.8	27.7	7
,,	450	34.4	38.3	39.7	42.8	0
,,	250	40.9	16.4	17.9	24.2	0

had been killed. A description of the chromosome aberrations regarded as characteristic of the action of true radiomimetic agents has already been given (Hendry, Rose, and Walpole, 1951). The increase over controls in the percentage of anaphases showing abnormalities of this type in tumour tissue from rats treated with the compounds in the doses shown is recorded in Table II. Mainly qualitative observations upon cytotoxic effects in the bone marrow and the results of tumour inhibition tests with the substances in question are also included.

As with compounds tested earlier and reported upon in Part I, there is an obvious correlation between tumour inhibitory activity and the capacity to induce in the tumour chromosome changes of the radiomimetic type. Only one compound in the present selection, namely, 1 : 4-pentadiene dioxide (226), which proved active in the latter sense, failed to produce a significant inhibition of tumour growth. The reason for this failure is not clear, but it may be that in the tumour growth test dosage was not pushed to the limit. Lack of material has so far prevented a repetition of the test.

Carcinogenic activity

Two of the more active inhibitors of tumour growth, namely, 1 : 3-butadiene dioxide (mixed isomers) and 4-vinyl *cyclo*hexene dioxide, have been tested for carcinogenic activity in rats and mice.

In rats, butadiene dioxide (0.4 per cent) dissolved in arachis oil was injected intraperitoneally in a dose of 2 mg. per 100 g. body weight twice weekly for six to seven weeks (13 doses) and in a dose of 1 mg. per 100 g. for the following six weeks (12 doses). Ten male and four female stock albino rats of average weight 125 g. were used and all were dead 18 (calendar) months from the start of treatment. In one male which died at 13 months (15–16 months old) large masses of a mixed-cell sarcoma were found in the peritoneal cavity, adherent to and infiltrating the stomach and body walls, and nodules of tumour tissue throughout the mesentery. A chondrosarcomatous mass was found enveloping the left lower ribs. Mixed cell sarcomata were seen in one testis and an unidentified epithelial tumour in the other. No tumour was found in any other rat in this group.

In mice the compound, dissolved in arachis oil or in acetone (A.R.), was applied repeatedly to the interscapular region of the skin. In one experiment 20 male stock mice received 1 drop daily (Saturdays and Sundays excepted) of a 10 per cent solution by volume in arachis oil for three months (66 doses). All the mice, which were mature at the beginning of the experiment, died within eight months thereof. No tumour was found in any animal, but loss of spermatogenesis was observed in the tubules of the testes in one. In a further experiment 20 male stock mice received two drops twice weekly of a 25 per cent solution of the compound in acetone for three weeks, followed by two drops of a 12.5 per cent solution twice during the week following. All died within 11 months of the commencement of the experiment. No tumours were observed in these mice, although areas of very pronounced infiltration with lymphoid cells, which may have been a leukaemic change, were found in the liver of one. Pyknosis of lymphoid cells was observed in the lymph nodes of several others.

In all these experiments a rather large proportion of the animals died during the early stages and further tests are being carried out with lower levels of dosage.

TABLE II

Growth-inhibitory and cytotoxic effects on the Walker tumour in rats of some epoxides and related compounds. *I* = percentage inhibition of tumour growth; A = percentage anaphases in excess of controls showing specific chromosome damage in tumour tissue from rats bearing the Walker tumour, twenty-four hours after the doses shown; B = true chromosome bridges; D = degenerating nuclei; E = "exploded" metaphases; F = chromosome fragments; P = pyknotic nuclei; S = "sticky" chromosome bridges

Serial No.	Compound	Tumour inhibition		Cytotoxic action			
		Total dose: mg. per 100 g., i.p.	*I*	Dose: mg. per 100 g., i.p.	Tumour		Bone marrow
					A		
221	4-Vinyl*cyclo*hexene dioxide	225	94	25 12.5	53 43	Some E and D	Some B, F, and S Some F and S
237	N: N-di-(2′: 3′-epoxy-*n*-propyl)-*p*-anisidine	170	86	30	76	Mostly showers of fragments. Many E	A few B, F, and S
231	Diethylene glycol diglycidyl ether	150	84	40	43	Some inhibition of mitosis. A few E	Some inhibition of mitosis. Some F and P
				20	—	Almost complete inhibition of mitosis. A few F	Some inhibition of mitosis. Some P
				10	39		A few P
232	Butane-1: 4-diol diglycidyl ether	120	74	20 10	62 38	Some E	A few F and B A few F and B
223	1: 4-Cyclohexadiene dioxide	150	43	50	0		Normal
213	Glycidyl methacrylate	150	40	30	1		A few P and S
226	1: 4-Pentadiene dioxide	250	29	100 50 25	41 42 2		B, F, and S A few P A few S
207	N-(2: 3-epoxy-*n*-propyl)-*p*-chloro-aniline	275	24	50	10		A few B
220	Safrole oxide	150	14	25	6		A few S
243	Ascaridole	20	7	4	1		A very few S
212	Glycidyl acrylate	3.5	0	0.5	2		A few S
219	α-Phenyl-α-bromomethyl-ethylene oxide	16	0	2	7	(Mean of 2 early tests)	A few S
				5	0		Normal
244	Ethylene glycol di-(3′-chloro-2′-hydroxy-*n*-propyl)ether	450	0	50	0		A few S
214	Glycidyl oleate	Untested		100	3		A few S
215	Glycidyl stearate—Crude Pure	,,		50 75	2 6		A few S A few S
202	α-Vinylnaphthalene oxide	,,		100	0		A few P

Vinyl *cyclo*hexene dioxide (5 per cent) dissolved in arachis oil was given intraperitoneally to ten male and four female stock albino rats in a dose of 25 mg. per 100 g., twice weekly for ten weeks. Seven months from the start of treatment mixed-cell sarcoma tissue was found widely disseminated in the peritoneal cavity of one male rat. One large area of the lung of this animal was infiltrated with tumour tissue. The sarcoma has been maintained for over 20 successive subcutaneous transplantations in stock rats. No other tumour has been seen in animals in this group, 6 of which are still alive 21 (calendar) months after the commencement of dosing. Loss of spermatogenesis has been observed in several of the males which have died and that at an age before the testes normally atrophy. Experiments in which the compound is being given subcutaneously to rats are in progress.

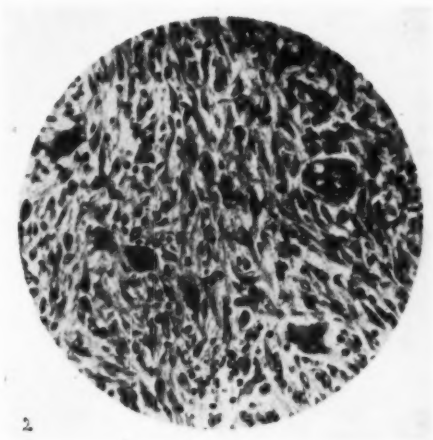
A much higher yield of tumours is obtained when the compound is applied to the skin of mice. Twenty stock albino males were painted in the interscapular region with one drop (ca. 16 mg.) of the compound, five times weekly for a total of twelve months. Skin papillomata began to appear within a few months, and many of these subsequently became malignant. The last of the mice died some 21 months after the start of treatment. Nine died without tumours, and, in two, papillomata which had developed during painting regressed after its cessation. Malignant tumours developed in the remainder as follows: Four had squamous cell carcinoma at the site of painting ; in one of these metastases were present in the lung, while in two lung adenomata showing no signs of malignancy were found. Three had mixed cell sarcomata in the tissues underlying the painted area. These sarcomata had ulcerated through at the surface, the epithelium being completely lost. In two mice both types of tumour were found together—a sarcoma ulcerating through at the surface bordered by carcinoma of the skin. In the lung of one of these animals a tumour of a third type, probably malignant, was found. Figs. 1 to 3 illustrate the appearance of the several tumours in this mouse.

The vinyl *cyclo*hexene dioxide used in the foregoing experiments was the commercial product and was contaminated with water insoluble material. In a further experiment a highly purified sample, completely soluble in water, was used. This sample was rather more toxic and rather more active in the production of specific cytotoxic effects in the Walker tumour than the original sample. A solution in acetone (3 parts) is being applied to the skin of mice at the rate of 1 drop per animal twice weekly. Loss of fur from the painted region was noted about one month after the start of treatment and the first papilloma made its appearance at two months.

Mutagenic activity (with Miss M. C. Frank)

Butadiene dioxide (mixed isomers) was tested for mutagenic activity upon the mould *Penicillium chrysogenum*. The latter was grown on agar slopes and aqueous suspensions of the spores, containing $3–6 \times 10^6$ per ml., were treated with the compound in aqueous solution. It proved to be rather toxic. At concentrations of 3 to 4 per cent it killed all the spores within 10 minutes at 24° C. ; at 0.5 per cent, the percentage survival after 10 to 60 minutes' exposure was 0.5 to 1. Concentrations of 0.5 and 0.25 per cent were used in the tests for mutagenic activity.

After treatment with the compound, the spore suspensions were plated on agar medium and the plates incubated at 24° C. The colonies which developed within



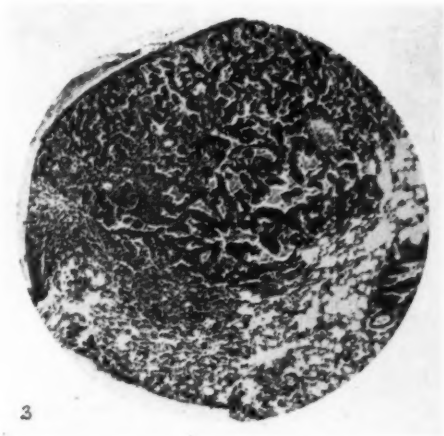


FIG. 1.—Section of tumours produced in mouse by painting the skin with 4-vinyl *cyclo*hexene dioxide. Field shows squamous-cell carcinoma of the skin at the periphery of a mixed-cell sarcoma (on left). H.E. × 77.

FIG. 2.—High-power view of sarcoma shown in Fig. 1. H.E. × 310.

FIG. 3.—Adenoma, possibly in early stage of malignancy, in an area of the lung of the same mouse. H.E. × 77.

a few days were examined macroscopically and mutants detected by changes in colour and habit of growth. Untreated spores produced green colonies having a characteristically raised centre. The mutants were of various hues, some being white or yellow and the majority various shades of yellow-green. As a matter of convenience the spore suspensions were sampled several times during treatment with the compound and colonies counted on all the plates showing a high degree of killing. It was impossible to forecast exactly what length of treatment would give suitable plates, i.e., plates containing 300 colonies or less. Control plates were also put up, as this strain of *P. chrysogenum* produces spontaneously a small number of mutants. The results may be summarized as follows:

Concentration of compound	Percentage of mutant colonies produced by 10–90 minutes' exposure		
%(w/v)	Exp. *a*	Exp. *b*	Mean
0.5	8.0	12.8	10.4
0.25		8.6	8.6
0 (control)	1.3	1.5	1.4

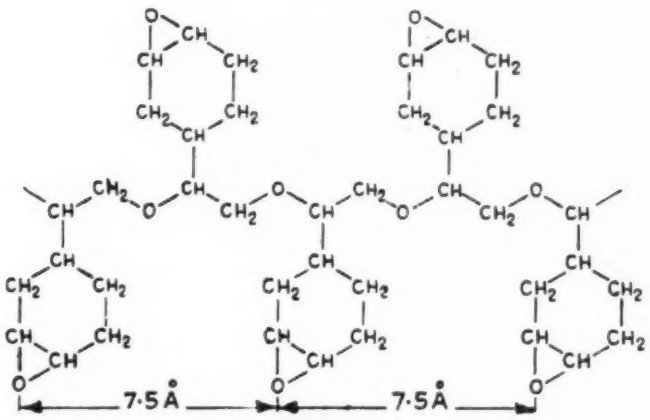
Some of the mutants were tested to see whether any change had occurred in penicillin productivity. This was found to vary over a wide range. The majority of the mutants gave poor yields, and it was evident from the types of growth obtained that many grew more slowly and less efficiently than the culture treated.

The proportion of mutants induced in *P. chrysogenum* by treatment with butadiene dioxide was rather lower than has usually been obtained with ultra-violet or *x* rays. Killing was about the same as that with ultra-violet rays but much more than with *x* rays. Similar results have been recorded for the sulphur and nitrogen mustards (cf. Bryson, 1948).

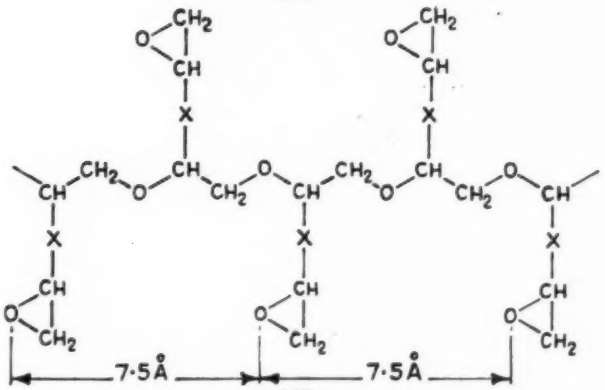
Discussion

At an early stage of this work it became clear that several considerations applicable to the methylolamides were equally relevant to the epoxides. In the first place, for significant inhibition of tumour growth in the rat, two epoxide groups in the same molecule were found to be necessary, and this suggested a biochemical equivalence of epoxide and methylolamide groups in accord with what is known of the actual chemical reactivity of the two types. Both, for example, show reactivity towards nucleophilic centres, the significance of which in the epoxide group was earlier pointed out by Rapoport (1948), who related it to the mutagenic activity shown by ethylene oxide and several derivatives in *Drosophila*. More recently, Ross (1950) has discussed the kinetics of this reaction in connexion with a range of epoxides also examined against the Walker tumour in rats, and it would appear that a certain minimal reactivity, measured *in vitro* with respect to the thiosulphate ion, must be exceeded before a compound of this type is growth-inhibitory. In agreement with our own findings, Ross observed that two such centres were required in the molecule (for a positive effect in the animal), and further suggested that an unequal reactivity was desirable, the more reactive grouping serving to anchor the molecule to a receptor, the second group then having more time to combine with another receptor. The studies of our Canadian colleagues, however, suggest an alternative explanation for the requirement of two functional groups, and one which takes into account an additional important chemical property of substances carrying the oxiran system. This property is ignored by Loveless and Revell (1949) in the simple cross-linkage hypothesis adduced by them to explain the cytotoxic effect of bis-epoxides.

It has been known for some time that ethylene oxide readily polymerizes to produce linear structures having the repeating unit [-O-CH_2-CH_2-], and this property persists in the more complex derivatives of this substance, including those carrying two oxide units. The tendency to polymerize is strongly conditioned by steric considerations, so that in 4-vinyl *cyclo*hexene dioxide (II), for example, it is possible to isolate a polymer (III) in which the second epoxide ring remains unaffected. This second oxide system is, however, the more reactive towards nucleophilic systems, so that the behaviour of (II), which is one of the more active (221 ; Table I) of the 24 *bis*-epoxides examined for inhibition of the Walker tumour, clearly suggests a separate function for each oxiran radical in these structures, the one being primarily concerned with polymerization and the other with combination with centres carrying active hydrogens such as occur in abundance in protein and, in particular, nucleoprotein systems. In the latter connexion, it may also be of



(III)



(IV)

importance to note that the dimensions of the -C-C-O- unit of the polyethylene glycol structure corresponds closely with those of the -C-C-N- unit of polypeptides, so that in a polymer of type (III) a reactive epoxide group occurs along each side of the chain at intervals of every two α-amino-acid residues. It will be recalled that a similar spacing was evident between the reactive groups in the polymeric structures proposed for the methylolamide series of tumour-inhibitors (Hendry, Rose, and Walpole, 1951), and a corollary of some importance follows from this observation in relation to the variation in structure permissible in the epoxide series. Reference to the Tables will show that tumour-inhibitory action was found in substances in which the two reactive centres are in close juxtaposition to one another as in the butadiene dioxides, and, at the other extreme, when they are some 15Å apart as in the diglycidyl ether of diethylene glycol (231). Examples of intermediate spacing occur in Table I. If the biological effects produced were due solely to bridges formed by individual molecules either along or between protein or nucleoprotein units, then it would be necessary to admit a wide range of permissible distances between the two points of attachment of each molecule to the latter. If, on the other hand, these cytotoxic agents function, not as individual molecules, but ultimately as polyreactive polymers of the generalized form (IV), then the distance between adjacent reactive groups is constant at about 7.5Å and theoretically independent of the dimension of the conjunctive group X.

The precise significance of the possible formation of these structures in relation to cytotoxic effects must necessarily be almost entirely a matter of speculation, but we suggest that, as in the methylolamide series discussed in the previous communication, the *bis*-epoxides diffuse in monomeric form into susceptible cells and there behave in one, or perhaps both, of two ways. One possibility is that they first polymerize to give structures corresponding to (IV), the side chains of which then react with protein or nucleoprotein of chromosomal origin to produce, by cross-linkage with multipoint attachment, the observed aberrations of mitosis and resultant effects upon cell proliferation. The second possibility involves initial reaction of one of the epoxide groups of each (monomeric) molecule with the cell component, followed by self-condensation of the free epoxide groups in the units so formed into a final polyethenoxy structure similar to that envisaged in the first suggestion. Either sequence of events would lead to a cross-linkage effect of unusual stability between, for example, two polypeptide units, one on either side of, and each joined to, the polymeric unit at a number of points. This would seem to be a significant elaboration of the concept mentioned by Loveless and Revell (1949) as a mechanistic explanation of cytotoxic effects of the nitrogen mustards and a bis-epoxide, namely, the formation of simple monomolecular link bonds between sister chromatids.

Postulation of cross-linkage in any form may, however, not be necessary, since it is certain that the function of protein or nucleoprotein within the dividing cell would be seriously impaired by the presence even of a single collateral polymer unit formed from the cytotoxic agent and held in position by numerous regularly spaced covalent bonds.

While the capacity of an agent to act in one or other of these ways may be necessary as a first condition for a specific disturbance of cell proliferation, reference to those sections of the Tables relating to the bis-epoxides indicates that tumour-inhibitory activity, which here must be referred to the sensitivity of the cells of the neoplasm consequent upon their characteristic high rate of proliferation, is dependent upon other structural requirements also. Thus, while no significant action on tumour growth in the rat was observed in substances other than those containing two oxiran radicals, not every example of the latter type was inhibitory. Some of the apparent anomalies could no doubt be explained on the basis of steric interference and rate of reaction, others on metabolic grounds, but such data are not yet sufficient for rational discussion. It is of interest, however, to note, for instance, the effect of the substituent in the benzene ring of the aniline derivatives 236, 237, 238, and 239, of which only the first pair, carrying *para* ethoxyl and methoxyl substituents respectively, were active. Similarly, although union of the oxiran groups through wholly aliphatic ether systems provides examples with high tumour-inhibitory activity (230, 231, and 232), the interposition of an arylether group (233, 234, and 235) in each case inactivates. The steric impediments, especially to polymerization, consequent upon poly-substitution of the epoxide ring are possibly responsible for the inactivity of limonene dioxide (222) and the dioxides, 227, 228, and probably 223.

An additional factor, which would condition access of the agent, is the extent to which polymerization may occur immediately after test animals have been dosed, since it might be expected that cell permeability to partially or wholly polymerized structures would be markedly reduced. Experience during the preparation of the

chemical specimens showed how widely this property would vary, since, while certain compounds appeared to have almost indefinite stability in the absence of catalysts, others passed into polymeric form before biological assays could be made.

The remaining compounds in the Tables call for little comment. As pointed out earlier, many of the simpler substances were selected as actual or potential precursors of active *bis*-epoxides, but were uniformly devoid of tumour-inhibitory action. Others, such as some of the *mono*-epoxides, were expressly included for comparison with closely related bifunctional derivatives (compare, for example, the active compound 221 (II) with 196, 197, 198, and 199).

Summary

1. Following upon the discovery of radiomimetic cytotoxic activity in a series of methylolamides a range of *bis*-epoxides with similar application as cross-linking agents in textile technology has been examined both for tumour-inhibitory and cytotoxic activity against the Walker carcinoma in rats. Some allied mono-epoxides have also been tested and the series of compounds investigated has been extended to include the parent olefins and dienes as possible epoxide precursors.

2. Marked activity in these tests has been shown only by certain members of the *bis*-epoxide class, in particular butadiene dioxide, vinyl *cyclo*hexene dioxide, certain N : N-diglycidyl derivatives of primary aromatic amines, and diglycidyl ethers of aliphatic diols. In every case, however, the margin between the lethal dose and that required to produce marked inhibition of tumour growth was too small to justify therapeutic application.

3. Intraperitoneal injection of vinyl *cyclo*hexene dioxide in arachis oil to stock albino rats in a dose of 25 mg. per 100 g. twice weekly for ten weeks gave at seven months a mixed cell sarcoma which has been maintained for over twenty successive subcutaneous transplantations in stock rats. A high yield of tumours has also been obtained in mice by skin painting. Evidence for carcinogenicity was not so strong with butadiene dioxide.

4. Butadiene dioxide produced mutations in a strain of *Penicillium*.

5. The theoretical implications of these findings are discussed. Evidence is adduced that the active agents may be linear polymers based on the repeating unit [-O-CH_2-CH-], in which the second epoxide ring in the residue R thus occurs along

|
R

the polymer chain at a constant step-distance. The reaction of this second epoxide system with cellular components, for example, protein or nucleoprotein, would result in a multipoint attachment which, it is suggested, might account for the cytotoxic effects observed.

We are indebted to Miss J. Gates and Miss D. Bonson for technical assistance.

References

Bryson, V. (1948). *J. Bact.*, **56**, 423.
Hendry, J. A., Rose, F. L., and Walpole, A. L. (1951). *Brit. J. Pharmacol.*, **6**, 201.
Loveless, A., and Revell, S. (1949). *Nature, Lond.*, **164**, 938.
Rapoport, I. A. (1948). *Doklady Acad. Nauk.*, **60** (3), 469.
Rose, F. L., Hendry, J. A., and Walpole, A. L. (1950). *Nature, Lond.*, **165**, 993.
Ross, W. C. J. (1950). *J. chem. Soc.*, 2257.
Walpole, A. L. (1951). *Brit. J. Pharmacol.*, **6**, 135.

Brit. J. Pharmacol. (1951), **6**, 256.

INHIBITION OF AMINE OXIDASE BY *ISO*THIOUREA DERIVATIVES

BY

F. N. FASTIER* AND JOYCE HAWKINS

From the Department of Pharmacology, University of Oxford

(Received February 28, 1951)

It is known that many compounds which contain the amidine group —C(:NH)NH_2 inhibit amine oxidase. Blaschko and Duthie (1945a), using a preparation from rabbit's liver, studied a number of homologous series of amidines and of amidine derivatives. They found that with increasing number of methylene groups there occurred at first a gradual increase in inhibitory activity. Thus with the mono-amidines of the series $CH_3(CH_2)_nC(:NH)NH_2$ inhibition was at a maximum at about $n = 10$, but with longer chains inhibitory activity fell off; it seems likely that this was due to the relative insolubility of the higher members, as suggested by Trim and Alexander (1949), for, in this series at least, surface activity and inhibitory potency reach a maximum at about the same member.

Some di*iso*thiourea derivatives were found by Blasckho and Duthie to be strong inhibitors of amine oxidase, but their study did not include any mono*iso*thioureas. We have now examined some forty amidine derivatives of the *iso*thiourea type, including thirteen members of the homologous series $CH_3(CH_2)_nS.C(:NH)NH_2$, as inhibitors of amine oxidase. At the same time we have studied the reversibility of the enzymic inhibition.

Material and Methods

The preparation of amine oxidase used was an acetone-dried powder of rabbit's liver suspended in phosphate buffer. The enzyme suspension contained 28.5 mg. of powder in 1 ml. 0.067 M-sodium phosphate buffer of *p*H 7.4. Soluble material was removed by centrifugation: after each of three washings fresh phosphate buffer was added to the sediment to bring the suspension up to its original volume. This procedure ensures a specimen of high enzymic activity and low enzyme blank (Blaschko and Hawkins, 1950).

Open manometers and conical flasks fitted with a side-bulb and potash tube were employed. The gas phase was oxygen and the bath temperature 37.5°. The main compartment of each flask contained 1.4 ml. of the liver suspension and 0.2 ml. of neutralized 0.1 M-semicarbazide hydrochloride; the potash tube 0.3 ml. of N-KOH; and the side-bulb 0.2 ml. of 0.1 M-tyramine hydrochloride and 0.2 ml. of either water or an aqueous solution of an *iso*thiourea salt.

The percentage inhibition of amine oxidase activity produced by an *iso*thiourea derivative was calculated from the amount of oxygen consumed during the first fifteen minutes after tipping in the flask containing enzyme and substrate (tyramine) only and the corresponding amount for the flask containing inhibitor also, corrections being made for the enzyme blank.

* Beit Memorial Fellow.

Most of the *iso*thioureas examined had been synthesized for earlier studies (Fastier and Smirk, 1943; Fastier, 1948). We are grateful to Abbott Laboratories and to Dr. Harold King for some of the compounds used.

RESULTS

Inhibitory action of isothiourea derivatives on the oxidation of tyramine

(*i*) *Mono-*iso*thioureas of general formula* $CH_3(CH_2)_nS.C(:NH_2)NH_2\}X$

Thirteen members of this series were examined, viz., the first ten ($n = 0$–9), the twelfth, fourteenth, and sixteenth ($n = 11$, 13, 15); X = Br except for S-methyl *iso*thiourea, the hydrochloride of which was used. Each salt was tested, if its solubility permitted, at a concentration of 10^{-3}M.

The percentage inhibitions produced by these *iso*thioureas are shown in Fig. 1, which illustrates the influence of chain-length on activity. It can be seen that

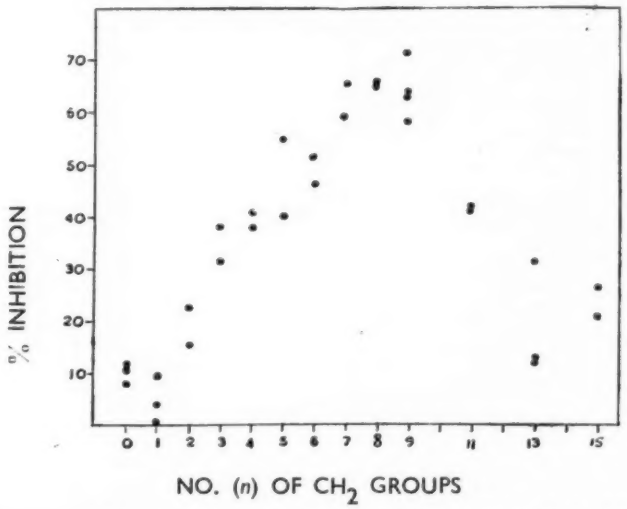


FIG. 1.—Inhibition of amine oxidase of rabbit's liver by S-alkyl *iso*thioureas of formula $CH_3(CH_2)_nS.C(:NH)NH_2$. Each dot represents one experiment. Inhibitor concentration of 10^{-3}M.

activity reaches a peak at about the tenth member ($n = 9$). The subsequent abrupt fall is probably determined by the sparing solubility of higher homologues in the aqueous phase. Even with the *n*-decyl derivative, it was noticed that some precipitation occurred when a 10^{-4}M aqueous solution, placed in the side-arm of the manometer flask, was diluted ten times with phosphate buffer.

(*ii*) *Di-*iso*thioureas of general formula* $Br^-\{NH_2(NH_2^+:)C.S(CH_2)_nS.C(:NH_2^+)NH_2\}Br^-$

Eight members of this series were examined, viz., the first six ($n = 1$–6), tenth, and twelfth ($n = 10$, 12). The two latter were not sufficiently soluble in the aqueous phase to provide a concentration of 10^{-3}M. Nevertheless, enough dissolved to produce almost complete inhibition of amine oxidase activity. The lower homologues tested produced far feebler inhibition at a concentration of 10^{-3}M.

Our results with this series confirm and extend those obtained by Blaschko and Duthie (1945a). The contrast between the relatively weak inhibitory activity of the

short-chain compounds and the much more pronounced inhibition caused by the two long-chain members is clearly shown in Fig. 2.

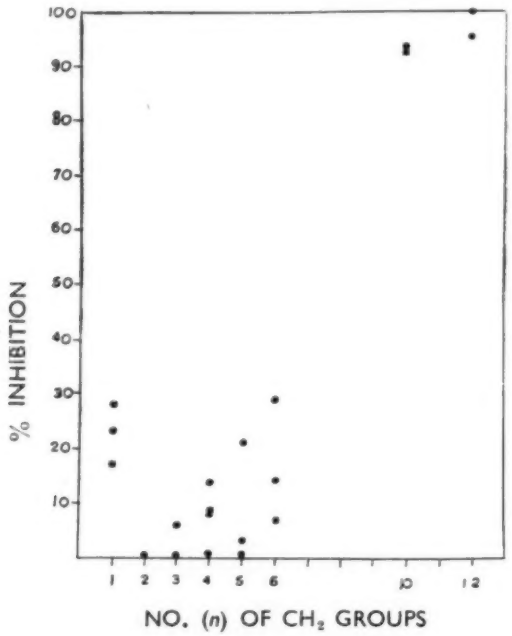


FIG. 2.—Inhibition of amine oxidase by SS′-alkylene di*iso*thioureas of formula $NH_2(NH:)C.S(CH_2)_nS.C(:NH)NH_2$. Each dot represents one experiment with an inhibitor concentration of 10^{-3}M.

(*iii*) *Other* iso*thioureas*

The introduction of an aromatic or alicyclic group into the side-chain of an S-alkyl *iso*thiourea may increase inhibitory activity considerably, as can be seen when the percentage inhibitions produced by the compounds listed in Table I are compared with those produced under the same conditions by S-methyl *iso*thiourea and its near homologues. Even the presence of substituents in the amidine group itself does not necessarily result in the loss of inhibitory activity. This is well shown when the percentage inhibitions produced by the three chlorobenzyl and the 2-pyridyl *iso*thioureas (Table I) are compared with those produced by the corresponding four derivatives of *NN′*-ethylene *iso*thiourea (2-thiol-4: 5-dihydroglyoxaline) (Table II). Four other N-substituted *iso*thioureas were tested, viz., S-*m*-xylyl-NN′-dimethyl *iso*thiourea hydrobromide, S-methyl-NN′-diphenyl *iso*thiourea hydrochloride, SN-trimethylene *iso*thiourea hydrobromide, and SN-(2-bromo-)trimethylene *iso*thiourea hydrobromide. The average percentage inhibitions which they produced were 96, 13, 5, and 0 respectively.

Reversal of inhibition of amine oxidase by washing

Using a different preparation of amine oxidase, Blaschko and Duthie (1945a) howed that the inhibition of the enzyme by pentamidine was not easily reversib

TABLE I

INHIBITION OF AMINE OXIDASE ACTIVITY OF RABBIT'S LIVER POWDER BY VARIOUS *iso*THIOUREA DERIVATIVES OF FORMULA $R.S.C(:NH_2)NH_2^+A^-$

The percentage inhibitions given are those obtained in individual experiments with an inhibitor concentration of 10^{-3}M

Structure of *iso*thiourea salt		Percentage inhibition
Radical	*Anion*	
benzyl	Cl	64, 55
β-phenyl-ethyl	Cl	67, 52
γ-phenyl-*n*-propyl	Br	77, 76
o-chlorobenzyl	Cl	89, 82
m-chlorobenzyl	Br	67, 52
p-chlorobenzyl	Cl	76, 64
o-bromobenzyl	Cl	99, 76
m-bromobenzyl	Br	75, 60
p-bromobenzyl	Br	74, 59
β-*cyclo*hexyl-ethyl	Br	81, 68
ε-*cyclo*hexyl-*n*-amyl	Br	75, 69
3-bromo*cyclo*hexyl	Br	47, 45
2-pyridyl	Br	30, 22
2-thienyl-methyl	Cl	50, 47

Since the preparation used in our own work was completely insoluble, it was easy to follow the removal of an inhibitor from the enzyme by repeated washings.

The method adopted was essentially the following: the enzyme was first treated three times with a concentration of inhibitor sufficient to cause a marked inhibition (60–90 per cent) of the enzymic activity. The enzyme was then washed three times with phosphate buffer and the inhibition remaining was determined; finally, the inhibition was again determined after another three washings.

The experimental details were as follows: In each experiment 350 mg. of rabbit's liver powder were suspended in 14 ml. phosphate buffer containing the inhibitor. The suspension was centrifuged at 740 *g* for 3 min. The sediment was re-suspended in another 14 ml. of the same solution, and the resulting suspension was centrifuged. The sediment from the second centrifugation was once again re-suspended and centrifuged, and the resulting sediment was again re-suspended in a fresh portion of the original inhibitor solution.

TABLE II

INHIBITION OF AMINE OXIDASE ACTIVITY OF RABBIT'S LIVER BY DERIVATIVES OF *NN'*-ETHYLENE *iso*THIOUREA

The percentage inhibitions given are those obtained in individual experiments with an inhibitor concentration of 10^{-3}M

S-substituent	Salt	Percentage inhibition
o-chlorobenzyl	Cl	95, 66
m-chlorobenzyl	Cl	66, 59
p-chlorobenzyl	Cl	78, 59
p-methoxybenzyl	Cl	63, 62
2-pyridyl	Br	90, 86

Another 350 mg. of the powder was treated in exactly the same way but with plain phosphate buffer. This specimen served as a control.

One-third of each specimen was retained after this treatment for the estimation of the initial enzymic activities. The remaining portions were centrifuged and each sediment was washed three times with plain phosphate buffer. After each centrifugation, phosphate buffer was added to bring the suspension up to the initial volume. Two suspensions were thus obtained, one which had originally been treated with inhibitor, the other a control which had been washed with plain phosphate buffer throughout. One-half of each suspension was kept for estimation of enzymic activity; the two remaining portions were subjected to three further washings in exactly the same way as before. One of the two suspensions finally obtained, that originally treated with inhibitor, had been washed six times in phosphate buffer before its enzymic activity was examined.

The amine oxidase activity of all six samples was estimated manometrically, either 0.4 ml. 0.05 M-tyramine or 0.4 ml. water being added to 1.4 ml. of the suspensions plus 0.2 ml. 0.1 M-semicarbazide.

Four members of the mono-*iso*thiourea series with differing inhibitory activities were examined first (n = 3, 5, 7, and 9). Suitable inhibitor concentrations were first found by trial; these were:

S-*n*-butyl *iso*thiourea ..	25×10^{-3}M	S-*n*-octyl *iso*thiourea ..	1.2×10^{-3}M
S-*n*-hexyl *iso*thiourea ..	6×10^{-3}M	S-*n*-decyl *iso*thiourea ..	0.2×10^{-3}M

The results illustrated in Fig. 3 show that the inhibitory effects of the four S-alkyl *iso*thioureas tested were always decreased by repeated washing of the treated powder,

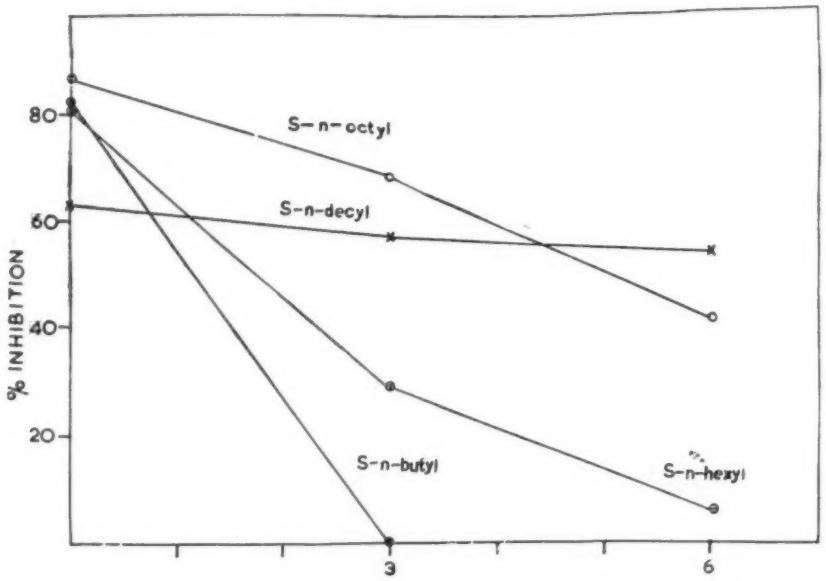


FIG. 3.—Reversal of the inhibition of amine oxidase, produced by S-alkyl *iso*thioureas, upon repeated washing with phosphate buffer.

but not equally. There is a clear gradation between S-*n*-butyl *iso*thiourea, which appears to have been completely removed from its site of action by three washings,

and S-*n*-decyl *iso*thiourea, the reversal of whose inhibitory effect was far from complete even after six washings. In this homologous series, the more tenaciously held compounds are those with the stronger inhibitory activity.

Similar experiments were also performed with S-benzyl *iso*thiourea and with pentamidine. When tested in a concentration of 1.2×10^{-3}M, S-benzyl *iso*thiourea produced 73 per cent inhibition of amine oxidase activity. The degree of inhibition was reduced to 23 per cent after three washings and to 8 per cent after six washings. Pentamidine, however, was held much more tenaciously. A concentration of 10^{-5}M in phosphate buffer sufficed to cause 92 per cent inhibition, and there was still 83 per cent inhibition after six washings.

Discussion

The experiments described in the first section of this paper show clearly that the *iso*thiourea derivatives have an affinity for amine oxidase, like so many other amidine derivatives. The experiments also show that the degree of inhibitory activity is determined not only by the presence of the basic amidine group but also by the hydrocarbon part of the inhibitor molecule.

Blaschko and Duthie (1945a) found that the diamidines included much more potent inhibitors of amine oxidase than the corresponding monoamidines. The same is true for *iso*thioureas. The mono*iso*thioureas examined by us were less active as inhibitors than the decamethylene and dodecamethylene di*iso*thioureas. The fact that compounds with two basic groups separated by a long hydrocarbon chain have a marked affinity for amine oxidase finds a parallel in the observation that the long-chain aliphatic diamines are good substrates of this enzyme (Blaschko and Duthie, 1945b; Blaschko and Hawkins, 1950).

It has already been pointed out (Fastier and Reid, 1948) with regard to certain pharmacological properties of *iso*thioureas that the increasing potency noted during the ascent of a homologous series does not necessarily mean that the molecule of a long-chain *iso*thiourea is more effective than that of a lower homologue at the site of action; the lower homologue may have a partition coefficient which is so much more unfavourable that many more molecules have to be present in the enveloping medium (in which the concentration is measured) in order to maintain the same number of molecules as the higher homologue at the " biophase " (Ferguson, 1939). If this is so, a long-chain *iso*thiourea should be washed away from its site of action less easily than a lower homologue; for supposing that a state of equilibrium is approached during each washing, the amount of *iso*thiourea removed will be determined largely by the partition coefficient. The results illustrated in Fig. 3 are therefore in accord with the view that the different degrees of inhibition of amine oxidase activity caused by a fixed dose of S-methyl *iso*thiourea and its homologues (Fig. 1) are due mainly to differences in their distribution between the aqueous " external phase " and the " biophase," in this case the enzyme.

Summary

1. Forty-four *iso*thiourea derivatives have been tested as inhibitors of the amine oxidase of rabbit's liver. Several of the compounds examined caused a marked inhibition of the enzyme. Of the S-alkyl mono*iso*thioureas examined, the decyl

member was the strongest inhibitor. Some of the long-chain alkylene di*iso*thioureas were even stronger inhibitors.

2. The reversibility of some of these inhibitions was studied by repeated washings and centrifugations; in the mono*iso*thiourea series the results show that the inhibition is most easily reversed with the low members of the series which are also the weakest inhibitors.

We are both grateful to Dr. H. Blaschko for helpful criticism and advice.

References

Blaschko, H., and Duthie, R. (1945a). *Biochem. J.*, **39**, 347.
Blaschko, H., and Duthie, R. (1945b). *Biochem. J.*, **39**, 478.
Blaschko, H., and Hawkins, J. (1950). *Brit. J. Pharmacol.*, **5**, 625.
Fastier, F. N. (1948). *Brit. J. Pharmacol.*, **3**, 19[illegible].
Fastier, F. N., and Reid, C. S. W. (1948). *Brit. J. Pharmacol.*, **3**, 205.
Fastier, F. N., and Smirk, F. H. (1943). *J. Physiol.*, **101**, 379.
Ferguson, J. (1939). *Proc. roy. Soc. B.*, **127**, 387.
Trim, A. R., and Alexander, H. E. (1949). *S.E.B. Symposia III, Selective Toxicity and Antibiotics*, p. 122. Cambridge University Press.

Brit. J. Pharmacol. (1951), **6**, 263.

THE MODE OF ACTION OF MORPHINE UPON THE INTESTINE

BY

E. M. VAUGHAN WILLIAMS* AND D. H. P. STREETEN†

From the Department of Pharmacology, University of Oxford

(Received February 23, 1951)

For more than sixty years differing views have been held about the action of morphine upon the intestine. The evidence was reviewed in detail in a recent paper (Vaughan Williams and Streeten, 1950), in which a new method of measuring intestinal propulsion was employed to demonstrate the relative potencies of analgesics on the activity of the bowel. Pethidine and amidone were found to be less active than morphine in retarding intestinal transport, in the ratio 75: 2.5: 1, the figures representing the number of molecules of *active base* of pethidine and amidone required to produce the same effect as one molecule of morphine base. In the same paper an explanation of the mode of action of morphine was offered, which resolved the apparent conflicts between the conclusions drawn by different workers employing a variety of methods. Further evidence is presented here which supports this explanation.

Many people have shown that morphine causes an increase of intestinal " tone "; that is to say, it induces a sustained contraction of a segment of intestine which will force fluid or air out of a recording balloon placed within the lumen. An increase in amplitude and frequency has also been observed in the so-called " peristaltic waves." Such changes in recorded intraluminal pressure have often been taken to imply that morphine would accelerate the propulsion of intestinal contents. This view was supported by the observation of Templeton and Adler (1940) that the force tending to drive onward a balloon placed in a Thiry-Vella loop, and held back by a thread attached to a spring, was temporarily augmented after injections of morphine; and by the results of Quigley, Highstone, and Ivy (1934), who observed that the passage of a lubricated rubber bolus, inserted into a Thiry-Vella loop, was at first accelerated by morphine, though the main effect of the drug was a prolonged slowing of the rate of its propulsion.

On the other hand, numerous *x*-ray studies of the movement of radio-opaque meals, observations of the expulsion of intestinal contents from fistulae in human patients, and measurements of the rate of passage of faecal pellets in rabbits, and of carbon suspensions in rats, had all shown that morphine reduced intestinal propulsive activity. In spite of the latter evidence, however, the view is still held by many surgeons and others that morphine improves the efficiency of intestinal propulsion, a belief based upon evidence obtained from intraluminal balloons or

* Schorstein Research Fellow, Oxford University.
† Nuffield Demonstrator in Pharmacology, Oxford University.

boluses, or derived from personal observations of intestinal activity during operations. Douglas (1949) noted that in dogs with Biebl loops morphine sulphate promoted " active contractions of the intestine," and Chesterman (1945) stated that " true peristalsis is increased for short periods " by morphine.

Measurements of the transport of fluid by cannulated Thiry-Vella loops (Vaughan Williams and Streeten, 1950) suggested an interpretation of the action of morphine which could reconcile these apparent contradictions. It was observed that the inhibition of propulsion caused by morphine occurred within a few minutes of injecting the drug, and that its onset was simultaneous with the expected increase in " tone," i.e., reduction in volume of the lumen of the loop. When the tone relaxed spontaneously for a brief interval, the transport of fluid was immediately resumed, only to cease again as soon as the lumen was occluded once more. The hypothesis was therefore put forward that the inhibition of intestinal transport produced by morphine was brought about by the very increase in tone which had been supposed by some previous workers to have improved it; and that the failure of propulsion was due, not to any paralysis or paresis of the intestinal muscle, but to a sustained contraction which closed off the lumen and impeded the development of co-ordinated propulsive movements. The results of Templeton and Adler (1940) and Quigley, Highstone, and Ivy (1934), already quoted, could thus be attributed to their employment of the unphysiological stimulus of inserting into the intestine solid boluses or balloons which forcibly dilated its walls. It is of interest that they noticed that after morphine more force was required to insert the boluses.

Methods

The preparation of the loops and the apparatus have already been described (Streeten and Vaughan Williams, 1951; Vaughan Williams, 1951). Briefly, a short segment of intestine, with a pedicle carrying nerves and blood vessels, was separated from the alimentary tract, the continuity of which was restored by a side-to-side anastomosis. Perspex cannulae were sewn into each end of the segment, and were fixed to the abdominal wall in such a way that, after the recovery of the dog from the operation, they gave permanent access to both ends of the loop, which itself lay within the abdominal cavity. To obtain records of intestinal motility, the cannulae were attached by water-tight joints to apparatus which enabled Tyrode solution to pass through the loop under controlled conditions of temperature and pressure, its rate of movement into and out of the loop being separately recorded. A comparison of the records enabled the volume, and thus the " tone," of the loop to be deduced.

Before it could be expelled from the loop, the fluid had to be raised by the contractions of the distal end to a height above the level of the reservoir from which it was running into the proximal end. Every drop of fluid transported having been lifted through a known and adjustable vertical distance, the rate of transport provided a quantitative estimate of the propulsive work done, measurable directly in gramme centimetres.

Results

In our previous paper (1950) it was suggested that an accelerated propulsion of solid boluses by the intestine and an increased pull exerted upon balloons after morphine was observed only because the intestinal wall was forcibly dilated by such objects. If this interpretation is correct, the complete inhibition of the transport of *fluid* induced by morphine should eventually be overcome if the dilating effect

of a bolus were imitated by increasing the pressure of the fluid at the entrance to the loop. The results of such a procedure are shown in Fig. 1, which depicts the activity of a loop (from the jejunum) about 12 cm. long. Tyrode solution was flowing in at a pressure of 10 cm., and had to be raised to a height of 11 cm. by

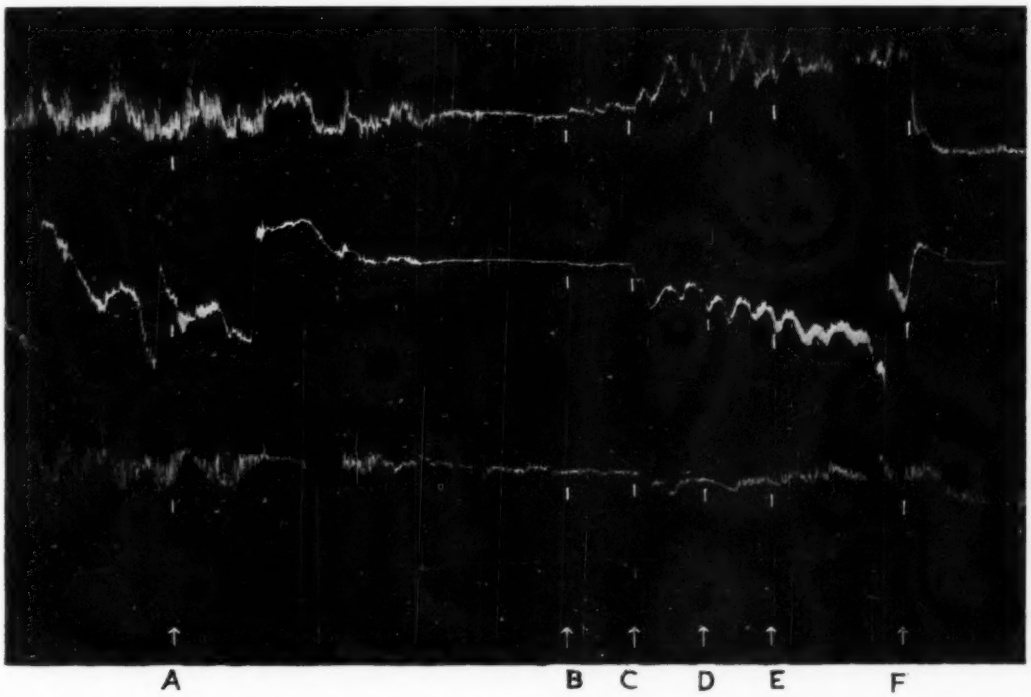


FIG. 1.—Activity of jejunal loop. Tyrode was flowing in at a pressure of 10 cm., and was pumped by the loop's contractions to a height of 11 cm. From above downwards: contractions of the proximal end of the loop; rate of flow into the proximal end; contractions of the distal end; rate of outflow from the distal end; time in minutes. At A, morphine sulphate (0.17 mg./kg.) was injected subcutaneously. At B, C, and D, the level of the inflow reservoir was raised to 13, 15, and 17 cm. Tyrode respectively. Not until the pressure was raised to 19 cm., at E, was the constricting effect of morphine overcome and the transport of fluid resumed. On the return of the pressure, at F, to 10 cm. once more, transport again ceased immediately.

the contractions of the loop before it could be expelled. The top tracing records the contractions of the proximal end, and every downward movement observed in the second tracing marks the admission of fluid into the loop. The third tracing depicts the contractions of the distal end of the loop, and each *upward* movement shown in the fourth tracing records the expulsion of fluid into an outflow recorder. The vertical straight lines in the second and fourth tracings mark the discharge, by a relay, of 10 ml. of fluid from the outflow recorder, and the simultaneous resetting of the inflow recorder by an equal volume of air. At A, 0.17 mg. morphine sulphate per kg. was injected subcutaneously. Five minutes later the rate of expulsion of fluid (fourth record) began to diminish, and shortly afterwards both inflow and outflow ceased altogether. Examination of the inflow and outflow records between the injection and the cessation of flow reveals that, whereas 11.8 ml. Tyrode had

been expelled from the loop, only 4.5 ml. had been admitted. The volume of the loop had thus diminished by 7.3 ml., indicating a closing down of its lumen.

At B, the pressure of the fluid in the proximal cannula was raised to 13 cm. In a normal loop under such circumstances (that is, with a pressure of 13 cm. at the proximal end and of only 11 cm. at the distal) fluid would have run passively through the loop. Under the influence of morphine, however, this did not occur, nor did it do so at C and D when the pressure was raised still further to 15 and 17 cm. respectively. As can be seen from the top record, the proximal end of the loop was stimulated to vigorous contractions by these high pressures; nevertheless, the lumen of the loop still remained closed, not a drop of fluid ran through the loop, and the contractions of the distal end of the loop (3rd tracing) were unaffected. When the pressure was raised to 19 cm. Tyrode, at E, the resistance of the contracted muscle was finally overcome, fluid ran both into and out of the loop, and the relay mechanism duly discharged 10 ml. from the outflow recorder. At F, the inflow pressure was returned to 10 cm., resulting immediately in the closure of the loop once more and a complete cessation of flow.

This result suggested that the belief that the inhibition of transport was due to simple closure of the loop in response to morphine might be confirmed equally well by the injection of drugs known to relax intestinal tone. In the experiment shown in Fig. 2, 0.13 mg. morphine sulphate per kg. was injected subcutaneously at A, with results similar to those in Fig. 1. At B, 0.042 mg. *l*-adrenaline per kg.

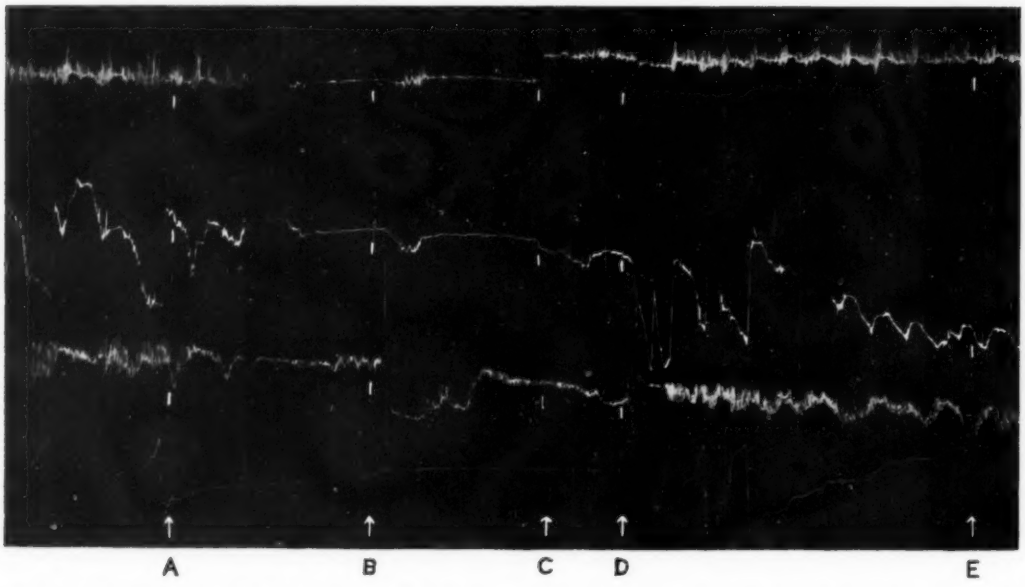


FIG. 2.—Activity of the same loop as in Fig. 1. Inflow pressure 10 cm. Tyrode, outflow sidearm 11 cm. above cannulae. At A, morphine sulphate (0.13 mg./kg.) was injected subcutaneously. At B, an intravenous injection of 0.042 mg. *l*-adrenaline per kg. was given, resulting in a dilatation of the loop, but not in the resumption of the transport of fluid. At C, the level of the inflow reservoir was raised to 12 cm., i.e., 1 cm. above the level of the outflow side-arm. At D, an intravenous injection of the same dose of adrenaline resulted in the flow of fluid through the loop. At E, the inflow pressure was returned to 10 cm. Tyrode.

was injected intravenously. Within a minute the tone of the intestine relaxed, and fluid ran into both the proximal end (2nd tracing) and back from the outflow tube into the distal end (3rd tracing). At the same time some contractions developed at the proximal end (top tracing). The effect was short-lived, however, and a few minutes later the situation was the same as before. At C the pressure at the proximal end was raised 2 cm. so that it was now 1 cm. above that at the distal end. At D the same dose of adrenaline was injected as at C. The fact that the inflow pressure was now higher than the outflow permitted fluid to run passively through the relaxed loop. At the same time, the presence of the fluid within the loop apparently stimulated the intestinal muscle, for the contractions were more vigorous and were maintained for a longer period than before. Eventually, however, as the effects of the adrenaline diminished, the lumen again closed down, and at E the passage of fluid once more ceased, when the pressure at the proximal end of the loop was reduced.

A similar effect, in an experiment on another dog, can be seen in Fig. 3. At A, 0.32 mg. morphine sulphate per kg. was injected subcutaneously, fluid transport

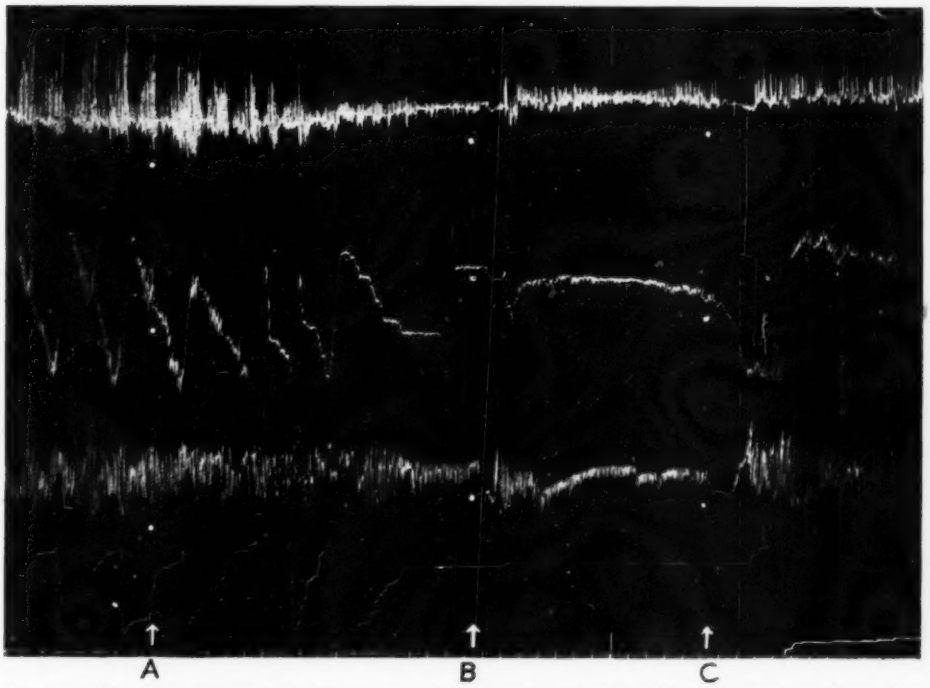


FIG. 3.—Activity of a different jejunal loop. Inflow pressure 9 cm. Tyrode, and outflow side-arm 10 cm. above cannulae throughout. At A, morphine sulphate (0.32 mg./kg.) injected subcutaneously. Intravenous injections were given of *l-nor*adrenaline: at B, 0.02 mg./kg. and, at C, 0.04 mg./kg. Fluid admitted to the loop during the relaxation caused by *nor*adrenaline was expelled again almost immediately.

ceasing completely within 15 min. The slight initial increase in the rate of transport was within the limits of variation normally encountered. The inflow pressure was 9 cm. Tyrode, and the outflow side-arm was 10 cm. above the loop throughout the experiment. (The break in the second record just before B represents a resetting

of the inflow recorder to avoid a subsequent overlap in the records.) At B, 0.02 mg. *l-nor*adrenaline per kg. was injected intravenously, causing a brief relaxation of the loop; not enough fluid entered to stimulate propulsion, however, and only two drops were expelled into the outflow recorder. At C, 0.04 mg. *l-nor*adrenaline per kg. was injected, resulting in the admission of 6.5 ml. from the inflow reservoir. This acted as a sufficient stimulus to promote the resumption of propulsive contractions, and some fluid was transported through the loop. Transport again ceased as the effects of the *nor*adrenaline diminished.

Atropine will also antagonize the effect of moderate doses of morphine, its effect being more prolonged than that of adrenaline or *nor*adrenaline. In Fig. 4, 0.32 mg.

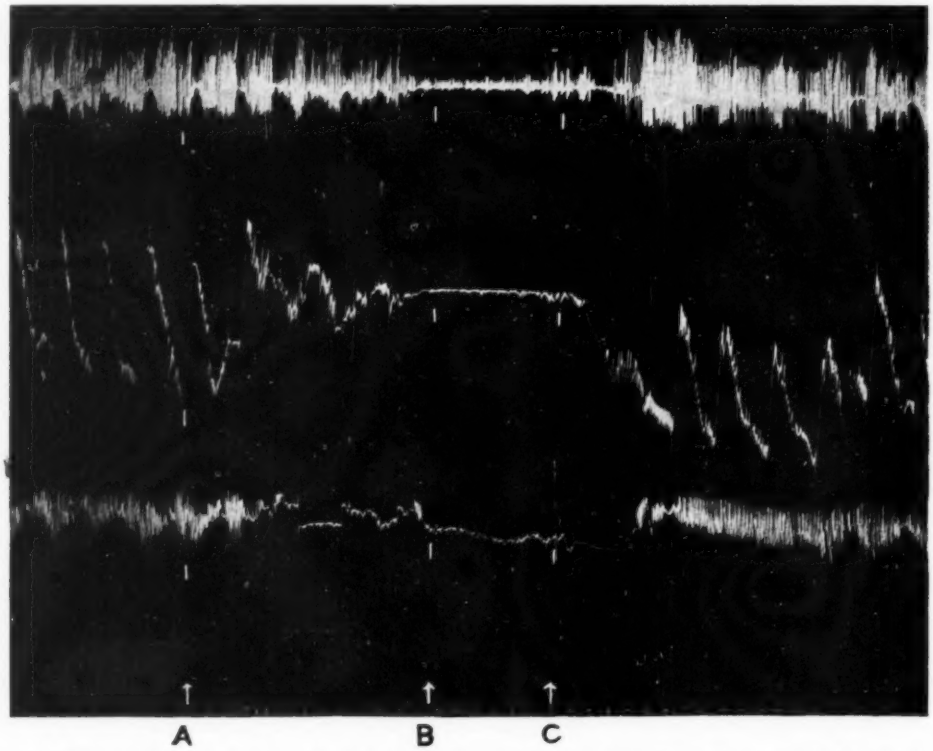


FIG. 4.—The antagonism to a moderate dose of morphine by atropine. At A, morphine sulphate (0.32 mg./kg.) injected subcutaneously. At B, the dog had a fit of retching. Under the influence of morphine, however, the big changes in intra-abdominal pressure resulting therefrom were not transmitted to the recording levers. At C, atropine sulphate (0.11 mg./kg.) was injected intravenously, and resulted in a resumption of fluid transport. Inflow pressure 9 cm., outflow side-arm 10 cm. above cannulae throughout.

morphine sulphate per kg. was given at A, and resulted in a fit of retching (B). In an untreated dog, although ordinary respiratory movements are not recorded on the tracings, such violent movements as these would have caused large excursions of all levers. Under the influence of morphine, however, the loop was contracted down, and presumably had either expelled all the fluid from its lumen, or had such a high tone in its walls that its contents were insulated from the big changes in

abdominal pressure associated with vomiting, for these were not transmitted to the recorders. At C, 0.11 mg. atropine per kg. was injected intravenously, and resulted in a resumption of intestinal transport, with a different rhythm but at approximately the same rate as before the morphine. If a larger dose of morphine was given, however, its effect was more powerful than that of the atropine, for it could not be antagonized by increasing the dose of atropine. This finding is illustrated in Fig. 5. The beginning of the tracing shows the activity of the same loop as that

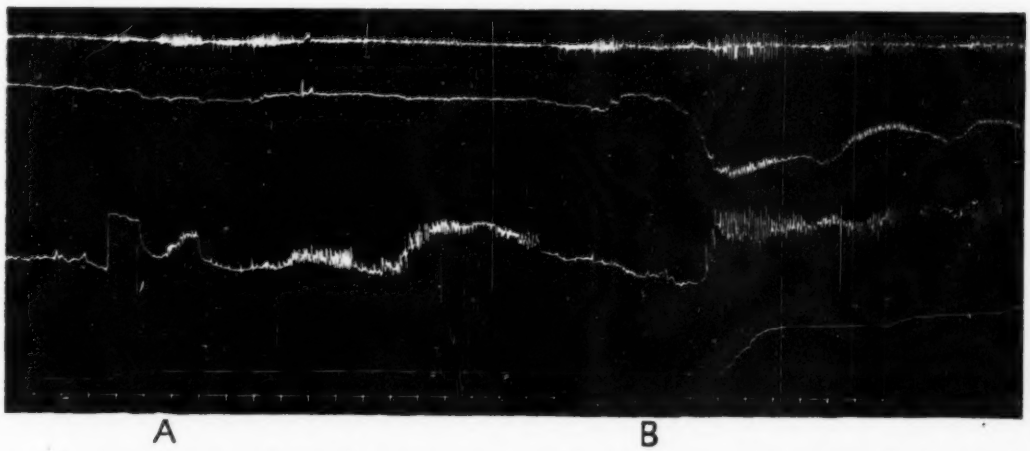


FIG. 5.—Twenty minutes before the commencement of the tracing, morphine sulphate (0.33 mg./kg.) had been injected subcutaneously and proved, in this dog (the same as that of Figs. 1 and 2), sufficiently large to produce an *in vivo* concentration such that adrenaline (0.04 mg./kg.) at A, and atropine (0.16 mg./kg.) at B, injected intravenously were now no longer able to antagonize appreciably the inhibition of fluid transport.

of Figs. 1 and 2 (which was more sensitive to morphine than that of Figs. 3 and 4), 20 minutes after an injection of 0.33 mg. morphine sulphate per kg. An intravenous injection of 0.016 mg. adrenaline per kg. had already been ineffective. At A, 0.04 mg. adrenaline per kg. was injected intravenously, and at B, 0.16 mg. atropine per kg. (the equivalent of approximately ten times the usual human dose), with little effect. It is clear that an antagonism to morphine by atropine could be expected to be demonstrated only if large doses of morphine were avoided, and this fact may explain the failure of some authors (Plant and Miller, 1926) to demonstrate such an antagonism, which was subsequently reported by many others (Gruber, Greene, Drayer, and Crawford, 1930; Oettel, 1935; Kanan, 1937; Myers, 1939; Adler and Ivy, 1940; Adler, Atkinson, and Ivy, 1942).

DISCUSSION

The results support the hypothesis that the inhibitory action of morphine upon intestinal propulsion is brought about by a prolonged contraction, which closes the intestinal lumen and prevents the development of co-ordinated propulsive contractions. This hypothesis reconciles the apparent conflict between the evidence, obtained from balloons and similar devices, that morphine increases intestinal activity, and the results from *x* rays of opaque meals and from other methods of measuring

intestinal *propulsion*, which have demonstrated that morphine retards the passage of contents along the bowel.

In a study of the influence of intraluminal pressure upon the transport of fluid by Thiry-Vella loops (Streeten and Vaughan Williams, 1951) it was shown that propulsive efficiency decreased when the jejunum was distended by pressures greater than 10 cm. Tyrode. Oettel (1934) stated that the intestinal contractions of the dog became " fatigued " when a balloon within the bowel was distended to 20–30 cm. H_2O for any length of time; and Krueger, Howes, and Gay (1935), employing pressures of 30–32 cm. H_2O in a similar preparation, found that the number of peristaltic waves during control experiments was " small in all dogs and equal to zero in many." A pressure at the inflow cannula of 10 cm. Tyrode was therefore regarded as the upper limit of " normal," and was used in the experiments presented here. Under such conditions morphine completely abolished the transport of fluid through the loops. This abolition was not due to any paralysis of the intestinal wall, for if fluid could be induced to enter the loop by raising the pressure at the inflow cannula, or by injections of relaxant drugs such as adrenaline or *nor*adrenaline, propulsive contractions were immediately resumed and the fluid expelled once more.

It is suggested, therefore, that the temporary acceleration of the passage of inserted boluses after morphine, and the increase in intestinal activity demonstrated by intraluminal balloons, has been demonstrated only as a consequence of the forcible distension of the intestinal wall by these unphysiological objects. Information about the distending pressures used in experiments with balloons has not always been published (exceptions being Plant and Miller (1926), 25–35 cm. water; Krueger (1934), 30–32 cm. water; Krueger, Howes, and Gay (1935), 30–32 cm. water), but it is noteworthy that, in the few balloon experiments in which a *reduction* of contractions after morphine was reported, lower pressures were employed, e.g., 15 cm. in the studies of Gruber, Brundage, DeNote, and Heiligman (1935).

In the experiments described in this paper moderate doses of morphine were used, ranging from 0.13 mg./kg. to 0.33 mg./kg., which for a human being of 60 kg. correspond to doses of 8 and 20 mg., or roughly $\frac{1}{8}$ and $\frac{1}{3}$ grain. That the effect of a moderate dose of morphine is mediated *in vivo* by some mechanism not present *in vitro* was suggested by the finding that the threshold concentration required to produce an effect *in vitro* was of the order of one hundred times the concentration calculated to be effective *in vivo* (Vaughan Williams and Streeten, 1950). The antagonism of moderate doses of morphine by atropine suggested that a cholinergic transmission was involved somewhere in the pathway mediating the effect. Large doses would, of course, raise the concentration of the drug in the extracellular fluid *in vivo* to levels which have been shown to be effective *in vitro*, and which could, therefore, exert a direct action upon the intestinal wall.

The mechanism of the action of moderate doses of morphine *in vivo* is far from clear and has already been discussed (Vaughan Williams and Streeten, 1950). It is of interest that the action upon the intestine of the drug amidone, which has been shown to have effects very similar to those of morphine, has been proved to be exerted through the vagi. In cross-circulation experiments, in which the head of a recipient dog was supplied with blood from a donor, Scott, Chen, Kohlsteadt, Robbins, and Israel (1947) showed that an injection of amidone into the donor circulation resulted in changes in intestinal activity (recorded by balloons) in the

recipient similar to those observed in a whole dog, although the recipient's trunk was connected to its perfused head by the vagus nerves alone. It is possible that morphine exerts its effect in a similar way. Against this must be set the evidence of Magnus (1906), Plant and Miller (1926), and Dreyer (1929), who found that denervation of the intestine did not abolish the effects of morphine. Magnus used doses of 30–40 mg. and Dreyer of 4 mg./kg., both of which would produce a concentration *in vivo* comparable to that effective *in vitro*, and which could, therefore, have produced a direct effect upon the intestinal wall. Plant and Miller, who used smaller doses of morphine (0.5 mg./kg.), admitted that their denervations were only partial. Parasympathetic denervation is known to result in the supersensitivity of the denervated effectors (Shen and Cannon, 1936), and the possibility cannot be ignored that the denervated receptors reacted to transmitter released by the fibres still left intact. None of these denervation experiments, therefore, can be taken as disproving the participation of the vagi in the effect of moderate doses of morphine on the intestine.

Appreciation of the mechanism of action of morphine has some clinical importance; in estimating, for example, the likelihood of its being beneficial in cases of intestinal distension or paralytic ileus. The evidence from experimental work is that, unless the intraluminal pressure is high, morphine has no stimulant action upon intestinal propulsion, but, on the contrary, abolishes it. If there is intestinal distension, and if the causes of such distension can be regarded as analogous to dilatation of the intestinal walls by boluses or balloons, then there is experimental support for the belief that morphine could temporarily facilitate intestinal propulsion. But, even under such circumstances, the phase of stimulation lasts for a period of minutes only, and is followed by a depression measurable in hours.

Such considerations apply, of course, to the effects of morphine upon the intestine only, and its depressant action there might well be thought to be outweighed by its beneficial effects in relieving anxiety and discomfort. A rational conclusion from the available evidence, therefore, would be that a single injection of morphine at the commencement of treating a case of paralytic ileus might be of value, but that continued administration of the drug could only result in an increasingly profound inhibition of intestinal propulsion, the return of which to normal would actually be prevented. It would seem preferable in such cases to use the other drugs now available, which, in doses of comparable analgesic potency, have been shown to have relatively less effect than morphine upon intestinal propulsion. Doses of both pethidine and amidone normally used for analgesia are below the threshold required to produce any observable inhibition of intestinal propulsion. Pethidine is the least active on the intestine, but its analgesic effect is qualitatively less good; i.e., increasing the dose does not compensate for its inability to relieve severe pain. Amidone, on the other hand, combines the advantages of great potency as an analgesic with relatively minor activity as an intestinal inhibitor.

Summary

1. Observations have been made of the tone and amplitude of contractions of cannulated Thiry-Vella loops in conscious dogs, and simultaneously of the rate at which the loops transport Tyrode solution under controlled conditions of temperature and pressure.

2. Morphine inhibits intestinal propulsion. The inhibition is caused by a closure of the intestinal lumen, not by any paralysis or paresis of the intestinal wall.

3. While under the influence of moderate doses of morphine, the loops will again transport fluid if they are distended by high pressures, or relaxed by adrenaline or *nor*adrenaline.

4. Atropine antagonizes moderate, but not large, doses of morphine.

5. The explanation offered of the mode of action of morphine is shown to reconcile apparent contradictions in previous evidence.

References

Adler, H. F., Atkinson, A. J., and Ivy, A. C. (1942). *Arch. intern. med.*, **69**, 974.
Adler, H. F., and Ivy, A. C. (1940). *J. Pharmacol.*, **70**, 454.
Chesterman, J. T. (1945). *Brit. med. J.*, **2**, 740.
Douglas, D. M. (1949). *J. Physiol.*, **110**, 66.
Dreyer, N. B. (1929). *J. Pharmacol.*, **36**, 477.
Gruber, C. M., Brundage, J. T., DeNote, A., and Heiligman, R. (1935). *J. Pharmacol.*, **53**, 120.
Gruber, C. M., Greene, W. W., Drayer, C. S., and Crawford, W. M. (1930). *J. Pharmacol.*, **38**, 389.
Kanan, M. A. (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 506.
Krueger, H. (1934). *J. Pharmacol.*, **50**, 254.
Krueger, H., Howes, H., and Gay, H. (1935). *J. Pharmacol.*, **55**, 288.
Magnus, R. (1906). *Pflüg. Arch. ges. Physiol.*, **115**, 316.
Myers, G. N. (1939). *J. Hyg., Camb.*, **39**, 391.
Oettel, H. (1934). *Arch. exp. Path. Pharmak.*, **175**, 588.
Oettel, H. (1935). *Arch. exp. Path. Pharmak.*, **177**, 317.
Plant, O. H., and Miller, G. H. (1926). *J. Pharmacol.*, **27**, 361.
Quigley, J. P., Highstone, W. H., and Ivy, A. C. (1934). *J. Pharmacol.*, **51**, 308.
Scott, C. C., Chen, K. K., Kohlsteadt, K. G., Robbins, E. B., and Israel, F. W. (1947). *J. Pharmacol* **91**, 147.
Shen, S. C., and Cannon, W. B. (1936). *Chinese J. Physiol.*, **10**, 359.
Streeten, D. H. P., and Vaughan Williams, E. M. (1951). *J. Physiol.*, **112**, 1.
Templeton, R. D., and Adler, H. F. (1940). *Amer. J. Physiol.*, **131**, 428.
Vaughan Williams, E. M. (1951). *J. Physiol.*, **112**, 32*P*.
Vaughan Williams, E. M., and Streeten, D. H. P. (1950). *Brit. J. Pharmacol.*, **5**, 584.

Brit. J. Pharmacol. (1951), 6, 273.

THE DURATION OF COCAINE ANAESTHESIA IN THE RABBIT CORNEA. A STATISTICAL EXAMINATION

BY

PETER A. YOUNG

From the Wellcome Research Laboratories, Langley Court, Beckenham, Kent

(Received December 28, 1950)

For over half a century the rabbit cornea has been employed as a site for the testing of local anaesthetic activity. Sixteen years ago, however, Copeland (1924) recognized the relatively large variability in the duration of action of any one treatment when measured on different rabbits. This variability has been subsequently described by many other authors, among them Coles and Rose (1929), Rider (1930), and Sinha (1936, 1939a). The duration of action on the cornea of locally applied anaesthetics is of sufficient importance, however, to warrant its measurement in spite of the large variations involved, provided that the results obtained are treated statistically. Chance and Lobstein (1944) asserted that " the test . . . has not withstood statistical examination," yet statistical grounds for its rejection have so far not yet appeared. It is the purpose of this paper to show that the test gives values which are statistically valid, although considerable numbers of animals are needed for useful results.

METHOD

Albino rabbits of various breeds have been used, each animal being employed repeatedly with at least one week between tests. Right and left eyes were used alternately for cocaine treatment at successive tests, the other eye receiving the compounds of unknown potency. Thus, between two cocaine treatments on one eye, a minimum of two weeks was allowed to elapse, during which time the eye would have usually received a dose of another synthetic compound. On the average the rabbits remain in use for about twelve months. A total of about 150 animals provided the data quoted below.

In the test the conjunctival sac is flooded with test solution, 1–2 c.c. as necessary, and the lower lid held over the upper for one minute. The criterion of anaesthesia is taken to be the absence of a blink reflex when the cornea is stimulated with a bristle mounted on a glass rod. This bristle, about 15 mm. long, is mounted adjacent to another, roughly half a millimetre shorter. Sufficient pressure may thus be exerted to bend the longer until its partner just contacts the eye, thus effecting some degree of standardization of the stimulus. Animals which are nervous or blink excessively without stimulation are not used for the tests. Observations on treated animals are made at five-minute intervals after the instillation of the drug until the blink reflex returns. The arithmetical mean of the times of the last negative and the first positive response is taken as the duration of anaesthesia.

RESULTS

Since the tests involve the repetition of treatments with cocaine, extensive data have accumulated over four years of routine testing.

The distribution of duration times

Until the beginning of 1950, cocaine solutions were prepared in saline, the concentrations most commonly used being 1, 2.5, and 5 mg. per c.c. Frequency distributions of duration times corresponding to these three levels were obtained from the records. The three frequency polygons are shown in Fig. 1. These

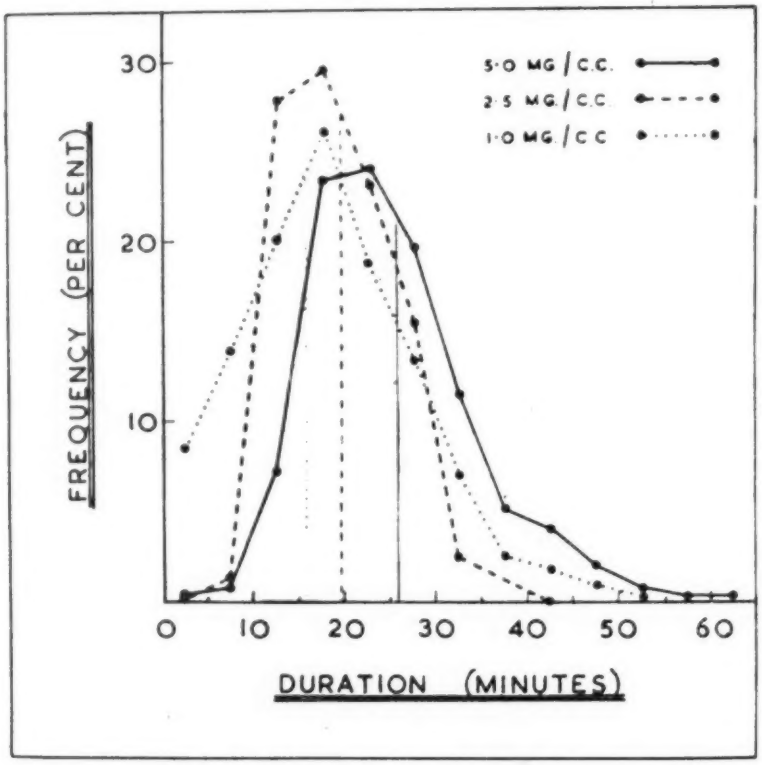


FIG. 1.—Frequency polygons of the durations of action of three concentrations of cocaine. The respective arithmetical means are indicated by the vertical lines.

values were not normally distributed with respect to time, and some simple normalizing transformation of the time values was sought. The best results were obtained with the logarithm of duration time, although frequencies at the lowest concentration were still not fitted by this means (see Table I). It was then observed that if the successive observations of duration were numbered 0, 1, 2, 3, etc., the corresponding frequencies for 1 mg./c.c. were distributed very nearly in a Poisson series. Although the total χ^2 measuring goodness of fit of this Poisson distribution showed significant deviation, this divergence was entirely contributed by the presence of thirteen values (2.3 per cent) which belonged to the upper tail of the distribution. If these are omitted, deviations from expectation have a probability of 0.96 (Table II). This relationship may, of course, be entirely spurious.

These findings suggest that above some threshold concentration (between 1 and 2.5 mg. per c.c.) duration times are log-normally distributed, while below the threshold the distribution is more skew. Since it is doubtful whether the threshold

TABLE I

Frequencies of observed (obs.) and expected (ex.) values of duration for three concentrations of cocaine in saline. Expected values derived on the basis of a log-normal distribution

Duration in minutes	Observed and expected frequencies for concentrations					
	1 mg./c.c.		2.5 mg./c.c.		5 mg./c.c.	
	Obs.	Ex.	Obs.	Ex.	Obs.	Ex.
2.5	48	46.8	0		2	
7.5	113	155.6	1	20.4	7	10.4
12.5	149	131.7	21		71	99.3
17.5	107	88.3	23	26.6	239	216.1
22.5	76	52.2	18	17.9	247	237.4
27.5	40	33.3	12		200	184.5
32.5	15	20.9	2	13.1	117	118.5
37.5	10	12.7	1		53	70.7
42.5	6	8.9			41	38.5
47.5	2				21	21.8
52.5	0				8	11.0
57.5	0	18.8			3	
62.5	1				4	12.8
67.5	2				8	
Total ..	569		78		1,021	
χ^2 (degr. freedom)	49.0 (9)		0.9 (3)		18.2 (10)	
p	<0.001		0.80		0.055	

TABLE II

Poisson distribution fitted to frequencies of duration times for 1 mg./c.c. cocaine in saline. o = observed; e = expected

Duration in minutes	All values			Omitting 13 highest values		
	o	e	$\frac{(o-e)^2}{e}$	o	e	$\frac{(o-e)^2}{e}$
2.5	48	46.7	0.036	48	45.6	0.126
7.5	113	116.8	0.124	113	114.1	0.011
12.5	149	145.9	0.066	149	142.6	0.287
17.5	107	121.7	1.776	107	118.9	1.191
22.5	76	76.0	0.000	76	74.3	0.039
27.5	40	38.0	0.105	40	37.1	0.227
32.5	15	15.8	0.041	15	15.5	0.016
37.5	10			8	7.9	0.013
42.5	6					
47.5	2					
52.5	0	8.1	20.544			
57.5	0					
62.5	1					
67.5	2					
Total ..	569			556		
χ^2 (deg. freedom)			22.692 (6)			1.910 (7)
p			<0.001			0.96

level is constant for all rabbits, we might expect to find some confusion of the two types of distribution at concentrations of about 1 to 2.5 mg. per c.c.

Some confirmation of these findings was available from results obtained since January, 1950. Since that date cocaine solutions were prepared in Sørensen buffer (*p*H 6), and a rather smaller series of results have been obtained with concentrations of 1 and 5 mg. per c.c. in this medium (Table III). An excellent fit is obtained by the log transformation at 5 mg. per c.c., and the Poisson series again gives a greater probability for the lower concentration.

TABLE III

Frequencies of observed and expected values for duration time for cocaine in buffer solution *p*H 6

Duration in minutes	Observed and expected frequencies for two concentrations				
	1 mg./c.c.			5 mg. c.c.	
	Obs.	Ex. (Poisson)	Ex. (log. normal)	Obs.	Ex. (log. normal)
2.5	16	12.6	17.5	1	10.3
7.5	29	27.8	34.5	7	
12.5	24	30.6	23.6	49	53 6
17.5	20	22.4	14.3	90	87.7
22.5	14	12.3	8.4	88	64.5
27.5	7			76	67.3
32.5	3	8.3	15.8	37	44.2
37.5	1			31	29.0
42.5				18	17.7
47.5				11	11.1
52.5				4	16.6
57.5				3	
62.5				1	
67.5				6	
Total ..	114	—	—	422	—
χ^2 (degr. freedom)		3.73 (5)	8.50 (5)		3.80 (9)
p		0.60	0.15		0.92

Further confirmation, although indirect, may be found in a paper by Rider (1930). Of results obtained with cocaine at 10 mg. per c.c. he wrote: " At least one-half of the rabbits tested have given a cocaine anaesthesia ten to fifteen minutes shorter than the value given " (i.e., the arithmetical mean of 27 minutes). This suggests that his results were distributed asymmetrically in the manner of log-normal distribution. More recently Sinha (1939a) gave histograms of duration times observed in groups of thirty to forty-five rabbits tested with three concentrations of cocaine. He observed that the coefficient of variation remained constant at about $\pm$ 20 per cent with mean values ranging between six and twelve minutes. This again indicates the applicability of a log-normal distribution.

Variation between rabbits

Rider, following the method of Coles and Rose (1929), adopted the practice of selecting for his experiments those animals which gave durations within a narrow

time range after the administration of 10 mg. cocaine per c.c. This procedure could have been partly justified if the variation between rabbits had been significantly greater than the experimental errors involved. In order to investigate the point, results on fifty rabbits, each of which was tested with cocaine at 5 mg. per c.c. four times in each eye, have been extracted and subjected to an analysis of variance (Table IV). The highly significant variance between rabbits confirms the earlier

TABLE IV

Analysis of variance of log duration times. Fifty rabbits tested four times in each eye with 5 mg. cocaine per c.c.

Source of variation	n	Mean square	F	p
Between rabbits	49	0.0793	5.55	<0.001
Between eyes	1	0.0181	1.27	>0.20
Between repetitions	3	0.1305	9.1	<0.001
Interactions:				
Rabbits × eyes	49	0.0224	1.56	Ca. 0.05
Rabbits × repetitions	147	0.0169	1.18	Ca. 0.20
Eyes × repetitions	3	0.0168	1.17	>0.20
Rabbits × eyes × repetitions (error) ..	147	0.0143		
Total variance	399	0.0252		

workers' grounds for eliminating those animals giving very high or low results. The question still arises, however, as to the justification of employing selected animals for comparative testing. While it probably would not affect the relative durations of different treatments, this would only be true in the absence of an interaction between the treatments and the rabbits. In a properly designed test the maximum of accuracy could be attained without selection of the experimental animals.

The analysis in Table IV also shows a highly significant mean square for " repetition." The four successive values of the geometric mean duration for fifty rabbits read once in each eye were : 1st 23.6, 2nd 21.8, 3rd 22.8, 4th 26.5 minutes, the grand mean being 23.6 minutes. Since no tendency was observed for values to continue to rise in rabbits used more than four times, it may be assumed that the variation observed is of a random nature, possibly influenced by the administration of other compounds, relatively more irritant, between successive trials with cocaine. It is to be concluded, therefore, that, within the limits of error indicated by the highest order interaction, absolute duration times are not repeatable. If consistent observations are to be obtained, the mean square for repetition must be considered as the error variance. This fact is discussed below at greater length.

As would be expected there is no over-all difference between the responses of right and left eyes, but the rather high mean square for the rabbits × eyes interaction indicates that there are slight differences between the results on the two eyes of individual animals. These differences would be minimized if the pairs of readings were made at the same time.

Since the log-normal distributions so far investigated (Tables I to III) consisted of a varying number of single observations on an undefined number of rabbits, it seemed desirable to check the distribution of the mean values of each rabbit in

the experiment analysed in Table IV. The number of animals was admittedly not large for the definition of a distribution, but reference to Table V shows a very satisfactory fit to be obtained by the log transformation.

TABLE V

Distribution of log-mean duration times for 50 rabbits (each value being the mean of four readings on each eye)

Log duration boundaries	Frequencies		
	Observed	Expected	
1.15–1.199	2	} 5.30	
1.20–1.249	5		
1.25–1.299	5	6.05	
1.30–1.349	9	8.70	$X^2 = 3.08$
1.35–1.399	11	9.90	6 d.f.
1.40–1.449	8	6.05	$p = 0.80$
1.50–1.549	4	}	
1.55–1.599	0	} 5.30	
1.60–1.649	1	}	

The relationship between concentration and duration

Since log duration is normally distributed for cocaine at concentrations above the threshold already described, we might expect to find a linear relationship between log duration and log dose. Sinha (1936) has shown that, while small quantities of cocaine solutions may give durations dependent on the volume administered, when the drug is applied in excess a maximum duration is obtained dependent only on concentration. In other words, with excess solution the log duration would be linearly related to log concentration. This was, in fact, Sinha's finding, giving the identity

$T = K.C^b$, which reduces to the more useful form of

$\text{Log } T = \text{Log } K + b \log C$,

Where T = duration time in minutes

C = concentration as percentage.

K is equivalent to the duration corresponding to Log $C = 0$, i.e., 1 per cent (w/v) or 10 mg. per c.c., and b the slope of the line.

In order to test this relationship with our own figures the results shown in Table VI were employed. These are the logarithmic means of all observations obtained at a variety of concentrations over a number of years. Since results with buffer solution were never significantly different from those with saline solution at the same concentration of cocaine, these have been pooled for this purpose. When the results

TABLE VI

Logarithmic means of duration times observed with varying concentration of cocaine

Concentration (mg./c.c.) ..	2.0	2.5	5.0	10.0	20.0	40.0	50.0	100.0
Mean duration (min.) ..	19.9	18.8	23.7	40.6	53.6	58.5	114.6	131.8
Number of observations ..	21	84	1,443	21	30	24	2	2

are weighted with their corresponding number of observations, analysis of the log values shows a highly significant linear regression mean square of 10.576, with a mean square for deviations from linearity of 0.137 (6 degrees of freedom). The latter is not likely to be significant, since the observations at different concentrations were made at different times. The regression coefficient b is equal to 0.467, standard error $\pm$ 0.053. The calculated value of K is 33.3 minutes (log K = 1.522).

In a subsequent publication Sinha (1939a) repeated his earlier observations, using rather larger groups of animals. He also tabulated some values of K and "n" (equivalent to our "b") derived from the work of other authors. These values, with some emendations and additions, are reproduced in Table VII with a list of

TABLE VII

DURATION (MINUTES) OF COCAINE ANAESTHESIA ON THE RABBIT CORNEA FROM VARIOUS AUTHORS

N.A. = No anaesthesia. I.A. = Incomplete anaesthesia. † = Values derived from best fitting regression lines. * = Omitted from computation of slope

Source	Duration (min.) for various concentrations (mg./c.c.) of cocaine						Slope (approx.)
	1.25	2.5	5.0	10.0	20.0	50.0	
				(*K*)			(*b*)
Young (general)†	13	17	24	33	45	70	0.47
Young (special experiment) ..		23	30	38	47		0.33
Sollmann (1918)	N.A.	N.A.	10	20	30		0.79
Regnier (1923)†			9	15	23		0.66
Copeland (1924)	I.A.	2*	13	18	30	>40	0.60
Schmitz and Loevenhart(1924)					28		
Cohen (1925)	10	17	23	42	46†		0.47
Coles and Rose (1929) ..					60		
Rider (1930)				27			
Uhlmann (1930)†	39	49	62	78			0.34
Bovet (1931)			14	19	31	60	0.64
Sinha (1936)†	—	9	13	18	27	—	0.54
Sinha (1939a)†	—	4	6	8	12	—	0.47
Hulpieu *et al.* (1940) ..					47		
Gilman *et al.* (1942) ..					20		

their sources. Sinha did not appear to recognize the linearity of the regression of log duration time on log concentration implicit in the formula he had derived. Indeed, he wrote in 1936 " the log concentration plotted against time . . . gives an approximately linear relationship." This statement he repeated in a later paper on the " Anaesthetic Action of Pyrazoline Compounds " (Sinha, 1939b). Plotting duration against log concentration he found that the slopes (of the " approximate straight lines ") for five compounds were dissimilar and inferred that the comparison of the concentrations producing anaesthesia of equal duration was of little significance. Such a sweeping conclusion might have been modified had he employed the log-duration/log-concentration relationship of his previous paper.

It will be seen from Table VII that there is a considerable variation in the duration quoted by different workers. The cause of the variation is discussed below. There is insufficient information to determine whether the slopes are more consistent between laboratories than are the duration times for any given concentration.

The method as a comparative assay

In an attempt to confirm the findings so far recorded and to assess the value of the technique for comparative testing, the following experiment was carried out. Sixteen rabbits were each tested four times at weekly intervals with four concentrations of cocaine in the right eyes and four concentrations of nupercaine in the left eyes. The animals were divided into four groups of four, and the order of treatments within the groups randomized in a latin square design. All drug solutions were in buffer (*p*H 6), and their identity was not known by the observer during the course of the experiments. The results obtained and their analysis are shown in Tables VIII and IX respectively.

TABLE VIII

THE DURATION-CONCENTRATION REGRESSION FOR COCAINE AND NUPERCAINE

The values quoted are expressed as the total number of stimulations producing no response at five-minute intervals after treatment. (Thus "5" corresponds to a duration of $5 \times 5 + 2.5 = 27.5$ minutes.) Each nupercaine group was made up from one rabbit from each cocaine group

Key to treatments:

C	Cocaine	2.5	mg./c.c.	F	Nupercaine	0.025	mg./c.c.
A	,,	5.0	,,	E	,,	0.050	,,
B	,,	10.0	,,	G	,,	0.100	,,
D	,,	20.0	,,	H	,,	0.200	,,

Group No.	Rabbit No.	Cocaine trials				Rabbit No.	Nupercaine trials			
		1	2	3	4		1	2	3	4
I	1	A7	C6	B6	D12	1	F7	H12	E6	G14
	5	B4	A1	D9	C1	2	E12	F2	G21	H25
	9	C6	D7	A4	B6	3	H22	G12	F0	E6
	13	D7	B7	C5	A7	4	G9	E7	H16	F0
II	2	A5	C4	B10	D12	5	F0	H6	E1	G6
	6	B14	A6	D11	C5	6	E14	F6	G13	H21
	10	C8	D13	A14	B13	7	H12	G10	F0	E3
	14	D9	B7	C9	A11	8	G5	E3	H14	F0
III	3	A4	C3	B5	D6	9	F0	H10	E0	G8
	7	B7	A8	D13	C3	10	E10	F5	G15	H24
	11	C7	D9	A4	B7	11	H20	G13	F4	E2
	15	D10	B14	C9	A13	12	G6	E2	H25	F2
IV	4	A5	C2	B4	D5	13	F0	H7	E2	G7
	8	B5	A3	D6	C1	14	E8	F0	G9	H13
	12	C6	D7	A5	B7	15	H14	G14	F9	E13
	16	D11	B8	C3	A6	16	G10	E8	H22	F1

The results confirm that the log-duration/log-concentration regression is linear for cocaine between 2.5 and 20 mg. per c.c. On the other hand, while nupercaine at strengths of 0.025 to 0.2 mg. per c.c. gave durations of the same order as the cocaine figures (see Fig. 2), there was a curvature of a low order of significance ($p = 0.05$–0.01). Moreover, the linear regression coefficients for the two drugs are significantly different, as are the log variances for error.

TABLE IX

THE DURATION-CONCENTRATION REGRESSION FOR COCAINE AND NUPERCAINE. ANALYSIS OF VARIANCE

Log values are taken throughout. Non-significant interactions have been pooled with the error sum of squares

Source of variation	d.f.	Cocaine			Nupercaine		
		Mean square	F	P	Mean square	F	P
Linear regression ..	1	0.8050	43.7	<0.001	9.5600	12.5	<0.001
Quadratic regression	1	0.0035	—	—	0.5059	6.5	0.05–0.01
Cubic regression ..	1	0.0000	—	—	0.0002	—	—
Groups	3	0.2133	11.6	<0.001	0.1266	1.65	0.20
"Rows"	3	0.1514	8.3	<0.001	0.5693	7.4	<0.001
Groups × rows ..	9	0.0597	3.3	0.01–0.001	0.2183	2.8	0.01
Trials (= "columns")	3	0.0212	1.2	>0.20	0.0140	—	—
Residual (ERROR)	42	0.0183			0.0767		
Linear regression coefficient (*b*)			0.333			1.148	
Standard error of *b*			±0.0503			±0.1029	
For the difference of these values of *b*			t = 7.12			p<0.001	
The ratio of nupercaine error variance to cocaine error variance			F = 4.19			p<0.001	

These three significant differences between the two drugs are sufficient to invalidate any single estimate of the relative potency of nupercaine in terms of cocaine which might otherwise have been obtained from the experiment. It would be reasonable to conclude that the two compounds differ qualitatively in their action on the cornea or in their interaction with it. Since this qualitative difference is due to the physico-chemical properties of the two compounds we might indeed find that the nature of the duration/concentration curve was a specific characteristic for every compound we could examine. It is likely, however, that unknown compounds could be classified as cocaine-like, nupercaine-like, and so on, the differences within each group not being detectable statistically by a reasonable number of experiments using this method.

Precision of the method

On the assumption that some compounds may have a qualitatively similar action to cocaine or nupercaine it is pertinent to consider the accuracy with which the activity of the unknown may be assessed relative to these standards. To demonstrate this we may compare the results obtained for 2.5 and 10 mg. per c.c. cocaine with those for 5 and 20 mg. per c.c., regarding them as pairs of dilutions of samples (*a*) and (*b*) respectively of cocaine. We find that the activity of the sample (*a*) is 99.6 per cent that of sample (*b*); to this deceptively accurate answer must be applied fiducial limits for p = 0.95 of 62–160 per cent. A similar examination of the nupercaine results gives us: sample (*a*) 0.025 and 0.1 mg. per c.c. = 100.7 per cent.

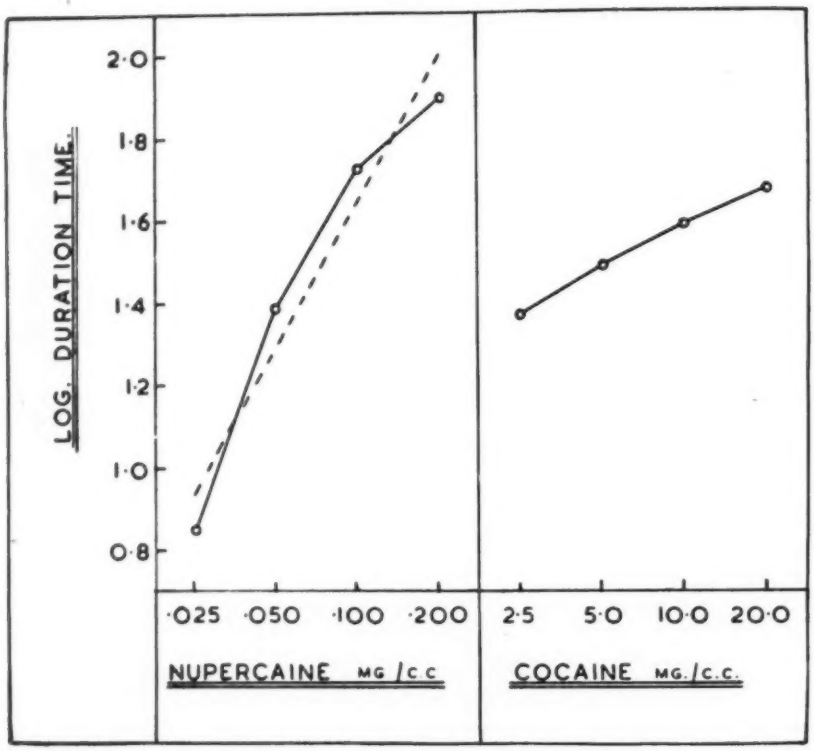


FIG. 2.—The regressions of log concentration and log duration for cocaine and nupercaine. The broken line for nupercaine is the fitted linear regression line.

as active as sample (*b*) 0.05 and 0.2 mg. per c.c., on the basis of linear regression. Fiducial limits are approximately 76–133 per cent. In neither example are the limits corrected for the variance of the regression coefficient.

It will be observed in Table IX that the mean square " trials " (i.e., the columns of Table VIII) is not significant against the error variance, where previously (Table IV) we obtained a significant repetition mean square. This discrepancy is probably accounted for by the fact that, although in the experiment " trials " are confounded with the rabbit × dose interaction, in the previously quoted results treatments with unknown compounds came between successive treatments with cocaine; thus, not only was there a possibility of the unknowns interfering with subsequent cocaine action, but the interval between trials with cocaine was at least twice as long in the earlier series as in the experiment.

By administering each treatment to each rabbit we are able to eliminate the rabbit to rabbit variation as " groups," " rows," and " groups × rows." The residual mean square may thus be used as an estimate of the error variance. It is interesting to note that for cocaine this value is of the same order as the final interaction mean square of Table IV.

The standard deviation of a single estimate of duration for each of the two drugs is shown in Table X. This is expressed both as percentage limits of the duration time observed and in terms of the corresponding percentages of concentration.

TABLE X

The error of a single estimate of duration time, as log increment and as percentage limits, in terms of time and concentration

	Cocaine		Nupercaine. Designed expt. (interaction error)
	General case (repetition error)	Designed expt. (interaction error)	
Standard deviation .. (% limits)	±0.3612 (44–230)	±0.1354 (73–137)	±0.2769 (53–189)
b	0.467	0.333	1.148
$\lambda = \frac{\text{s.d.}}{b}$ .. .. .. (% limits)	±0.7734 (17–593)	±0.4066 (39–255)	±0.2412 (57–175)

The latter values are obtained from the logarithmic value λ, which is given by the expression

$$\lambda = \frac{\text{standard deviation}}{\text{linear regression coefficient } (b)}$$

For comparison, similar values are listed derived from the general regression of Table VI, with a variance equivalent to the mean square for repetition found in Table IV. The advantage of a designed experiment will be seen by the great reduction in the value of λ compared with the general term.

Discussion

The greatest source of variation in results is undoubtedly inherent in the method, namely, the elicitation of the blink reflex. Many kinds of instrument have been employed as a stimulator. Leopold Ther (1949) quotes Kruchmann in 1895 and Schlüter in 1907 as using a bristle or Von Frey hair. This type was employed by Regnier (1923) and later by Bovet (1931), as well as by ourselves. Cohen (1925) used a toothpick tipped with cotton-wool. Most of the other authors quoted employed a rather more substantial stimulator following the lines of the blunt pencil point of Sollmann (1918). There is little evidence to suggest, however, that the choice in this respect has any consistent effect on the durations observed (see Table VII); nor can any conclusion be arrived at concerning its effect on the variability of results. Several workers have claimed an accuracy of the order of ± 20 per cent, but detailed analyses are not given.

It has commonly been observed that between the stages of no anaesthesia and complete blocking of the reflex there is a phase of partial anaesthesia which has two characteristics:

(1) the bristle applied to one point on the cornea may produce no response, but reapplied to another point may do so; and

(2) the response may be in the form of a slow closure of the eyelids, compared with the rapid movement of a normal untreated eye.

Regnier has claimed that the degree of anaesthesia may be estimated by the number of rapidly repeated stimuli required to produce a response. If this is

the case, since a bristle thus employed could not repeatedly fall on the same point of the cornea, we could not distinguish between an estimation of the degree of anaesthesia and the distribution of sensitive areas inferred in (1) above. Thus, while the bristle has the advantages of being less visible to the rabbits and of causing the minimum damage to the cornea, with a single stimulation we cannot guarantee to define the excitability of the whole area of the cornea. The response when produced may still be indistinguishable from a chance slow movement of the eyelids. The assessment of presence or absence of anaesthesia is then a highly subjective operation requiring considerable practice.

The relationship ($T = K.C^b$) discovered by Sinha appears to be true for cocaine in the concentration range 2.5 to 20 mg. per c.c., and may hold good up to concentrations of 50 mg. per c.c. or higher. It would seem unlikely that K should possess a universally valid value, but whether b varies significantly is a matter for conjecture. Nevertheless, under a given set of conditions, the log-duration/log-concentration relationship may validly be used for the comparison of anaesthetic compounds. That such comparisons may not always be resolved into a single estimate of relative potency has been demonstrated for cocaine and nupercaine, but it is not the purpose of this paper to investigate the mechanism by which the qualitative difference between these two drugs exerts itself; the nature of their difference is, however, of no small pharmacological importance. As Sinha rightly asserted, the fractional value of b for cocaine involves the use of relatively high concentrations of the drug for a given extension of its duration of action. Thus to double the duration we should require approximately four times the concentration. On the other hand, within the limits of the single experiment outlined above with nupercaine, the value of b is approximately unity. In other words to increase the duration by twofold would only require a twofold increase of concentration. It must be pointed out, however, that the curvature of the nupercaine regression line (Fig. 2) suggests that this property may not hold for very high values of duration and concentration. The nature of the regression clearly becomes of prime importance in assessing the relative merits of compounds to be employed for surface anaesthesia.

Summary

The duration times for cocaine solutions at concentrations of 2.5 mg. per c.c. or greater are log-normally distributed. The absolute duration times for cocaine vary significantly both between rabbits and between treatments on the same rabbits when the animals are tested alternately with cocaine and compounds of unknown potency.

From 2.5 mg. per c.c. to about 50 mg. per c.c. the log duration is linearly related to log concentration for cocaine.

Nupercaine at 0.025 to 0.2 mg. per c.c. gave a different regression of log duration on log concentration from that of cocaine.

This difference and the precision of the method are discussed.

I should like to acknowledge my indebtedness to Dr. A. C. White and Mr. A. F. Green for advice and access to their accumulated data on corneal anaesthesia, to Miss H. Muller, who carried out the pharmacological tests, and to Mr. J. B. Attlesey for assistance in the computations.

References

Bovet, D. (1931). *Arch. int. Pharmacodyn.*, **41**, 103.
Chance, M. R. A., and Lobstein, H. (1944). *J. Pharmacol.*, **82**, 203.
Cohen, S. J. (1925). *J. Lab. clin. Med.*, **11**, 174.
Coles, H. W., and Rose, C. L. (1929). *J. Lab. clin. Med.*, **15**, 239.
Copeland, A. J. (1924). *Brit. med. J.*, **2**, 241.
Gilman, A., Goodman, L., Thomas, J. M., Hahn, G. A., and Prutting, J. M. (1942). *J. Pharmacol.*, **74**, 290.
Hulpieu, H. R., Kitchel, J. H., and Weatherby, J. H. (1940). *J. Pharmacol.*, **68**, 395.
Regnier, J. (1923). *Bull. Sci. Pharm.*, **30**, 580, 646.
Rider, T. H. (1930). *J. Pharmacol.*, **34**, 329.
Schmitz, H. L., and Loevenhart, A. S. (1924). *J. Pharmacol.*, **24**, 159.
Sinha, H. K. (1936). *J. Pharmacol.*, **57**, 199.
Sinha, H. K. (1939a). *J. Pharmacol.*, **66**, 42.
Sinha, H. K. (1939b). *J. Pharmacol.*, **66**, 54.
Sollmann, T. (1918). *J. Pharmacol.*, **11**, 17.
Ther, Leopold (1949). *Pharmakologische Methoden.* Stuttgart: Wissenschaftl. Verlagsges.
Uhlmann, Fr. (1930). *Arch. int. Pharmacodyn.*, **36**, 253.

Brit. J. Pharmacol. (1951), **6**, 286.

*NOR*ADRENALINE OUTPUT IN URINE AFTER INFUSION IN MAN

BY

U. S. VON EULER AND R. LUFT

From the Physiological Department, Faculty of Medicine (Karolinska Institutet), and the Endocrinological Department, Serafimerlasarettet, Stockholm

(Received March 5, 1951)

*Nor*adrenaline is normally excreted in human urine in amounts of some 20–40 μg. per day (Kroneberg and Schümann, 1950 ; Euler and Hellner, 1951a). Under certain conditions the output is increased, e.g., during heavy muscular work (Euler and Hellner, 1951b) and in phaeochromocytoma (Engel and Euler, 1950), where it may attain values of more than 2,000 μg. per day.

In order to study the excretion in relation to administered *nor*adrenaline in man we have infused the hormone at a fixed rate intravenously and analysed the urine for *nor*adrenaline and adrenaline.

Six experiments were carried out on four subjects. Cases 1 and 2 were patients with morbus Addison and subjects 3 and 4 were healthy medical students, whose co-operation is gratefully acknowledged. *dl-Nor*adrenaline hydrochloride, dissolved in normal saline, was infused intravenously at a rate of 16.4–28 μg. per min. Urine was collected for a period immediately before infusion, during infusion, and, in two experiments, immediately after infusion.

The catechol amines from the urine were prepared and analysed according to the method of Euler and Hellner (1951a). The recovery of *nor*adrenaline and adrenaline can be estimated at about 70 per cent.

In order to split conjugated *nor*adrenaline and adrenaline the urine was hydrolysed at *p*H 2 for 20 min. at 100° before the absorption of the catechol amines. 1 ml. 20 per cent aluminium sulphate was added per 100 ml. urine ; 0.5 N-NaOH was then added dropwise under continuous stirring until *p*H 7.6 was reached and the aluminium hydroxide formation was complete. The precipitate was allowed to settle and then filtered off, washed twice with distilled water, and dissolved in 2 N-H_2SO_4. The solution containing the dissolved precipitate was then adjusted to *p*H 3.5 with 0.5 N-NaOH. Four volumes of equal parts of ethanol and acetone were then added in order to precipitate the salts. The solution was left in the refrigerator for some hours, filtered, and the filtrate evaporated *in vacuo* to a small volume. The final extract was made up to correspond to 5–25 ml. original urine per ml. and *p*H adjusted to 4. This extract could be tested directly biologically and showed no actions other than those of the catechols present.

The biological estimation was made on the cat's blood pressure and on the fowl's rectal caecum, according to the method described by Euler (1949).

The assay of the urinary extract was made in terms of *l-nor*adrenaline and *l*-adrenaline hydrochloride. Since the activity of the *dl-nor*adrenaline is approxi-

TABLE I

Subject	Infused *dl-nor*adr. μg./min.	Time min.	Vol. ml.	Excreted		% adr.	Extra output of *l-nor*adr. μg./min.	% output of infused *nor*adr.
				l-adr. μg./min.	*l-nor*adr. μg./min.			
1*a*. (D.):								
Before infusion		115	102	0.0077	0.0178	30		
During infusion	16.4	35	74	0.019	0.138	12	0.120	1.5
1*b*. (D.):								
Before infusion		50	134	0.012	0.0094	57		
During infusion	16.7	50	205	0.0115	0.201	5.5	0.192	2.3
2*a*. (S.):								
Before infusion		33	80	0.011	0.0213	33		
During infusion	17.7	65	345	0.0125	0.314	3.8	0.293	3.3
2*b*. (S.):								
Before infusion		90	590	0.0086	0.015	36		
During infusion	16.4	60	265	0.009	0.217	4	0.202	2.5
3. (K.L.):								
Before infusion		78	56	0.0079	0.0255	24		
During infusion	24.6	65	360	0.0266	0.266	9	0.240	2.0
After infusion		45	116	0.016	0.071	18		
4. (A.K.):								
Before infusion		79	380	0.0212	0.060	26		
During infusion	28.0	61	600	0.053	0.413	11	0.353	2.5
After infusion		59	140	0.027	0.056	33		

Figures are given in terms of *l-nor*adrenaline hydrochloride.

mately one half of that of *l-nor*adrenaline the former figures have been halved for the computation of the output percentage. The error introduced by this approximation is small. The results are given in Table I.

In all experiments there was about a tenfold increase in the amount of *nor*adrenaline excreted per minute during the infusion. From the increased output of *nor*adrenaline compared with that normally found it appears that 1.5–3.3 per cent of the amount administered per minute was recovered from the urine. The recovery percentage indicates that the inactivation was rather effective. In the period after termination of the infusion the *nor*adrenaline figure was still distinctly increased, although considerably less than during infusion.

Discussion

*Nor*adrenaline infusion of the same order of magnitude as in the present experiments, viz., some 0.1–0.2 μg. *l-nor*adrenaline per kg. per min., generally causes a rise of blood pressure (Goldenberg, Pines, Baldwin, Greene, and Roh, 1948, and others). Such an effect was also noted in the present experiments. The relatively large increase in *nor*adrenaline excretion indicates that the normal inflow of *nor*adrenaline into the circulation is much lower than that administered. Unfortunately it has not been possible to obtain reliable figures for the blood or plasma

concentration of *nor*adrenaline during the infusion. Such a figure would have permitted determination of the inactivation rate and perhaps also have given some idea of the normal *nor*adrenaline concentration in plasma. In human whole blood a figure of 1–2 μg. *l-nor*adrenaline per 100 ml. has been observed (Euler and Schmiterlöw, 1947), but this may be too high owing to the fact that *nor*adrenaline is taken up by the blood corpuscles (Bain, Gaunt, and Suffolk, 1937).

It is of interest to compare the excretion of *nor*adrenaline during infusion at a known rate with the excretion determined in cases of phaeochromocytomas, in which condition the excretion has amounted to 0.3–1 μg. per min. (Engel and Euler, 1950). The figure 0.3 μg. is about equal to the highest obtained during infusion in the present experiments and corresponds to an inflow of some 9 μg. *l-nor*adrenaline per min. or about 0.15 μg. per kg. per min. This amount generally raises the systolic blood pressure some 30–40 mm. in normal subjects, but may have a much stronger action in hypertensives (Goldenberg *et al.*, 1948).

If the relation between input and output is similar during normal conditions, without extra administration of *nor*adrenaline, as in the case outlined above, the normal output in the urine of some 0.02 μg. *l-nor*adrenaline per min. during rest would mean that about 0.6 μg. *l-nor*adrenaline per min. was entering the general circulation. Such a figure is not incompatible with the experimental results of Goldenberg and associates, who found 0.05 μg. per kg. per min.—i.e., 3 μg. per min. in a 60-kg. person—causing a slight increase in blood pressure.

The figures for adrenaline do not show any consistent changes during the infusion of *l-nor*adrenaline, which suggests that infused *nor*adrenaline is not converted into adrenaline to any appreciable extent in the body.

In the period immediately following the infusion there was still an increased output of *nor*adrenaline, although to a much smaller extent than during the infusion. This excretion may be explained either on the assumption that the bladder was not completely emptied at the end of the infusion or that some *nor*adrenaline had been taken up by the blood or by some other tissues and was being gradually liberated.

Summary

Infusion of 16.4–28 μg. *dl-nor*adrenaline per min. in two patients and two healthy subjects increased the *nor*adrenaline excretion about tenfold, without producing marked changes in adrenaline excretion.

1.5–3.3 per cent of the infused amount was found in the urine per unit of time.

From the relation of administered and excreted *nor*adrenaline it is inferred that normally some 0.5–1 μg. *l-nor*adrenaline per min. reaches the general circulation.

References

Bain, W. A., Gaunt, W. E., and Suffolk, S. F. (1937). *J. Physiol.*, **91**, 233.
Engel, A., and Euler, U. S. v. (1950). *Lancet*, **259**, 387.
Euler, U. S. v. (1949). *Acta physiol. scand.*, **19**, 207.
Euler, U. S. v., and Hellner, S. (1951a). *Acta physiol. scand.* (in the press).
Euler, U. S. v., and Hellner, S. (1951b). *Acta physiol. scand.* (to be published).
Euler, U. S. v., and Schmiterlöw, C. G. (1947). *Acta physiol. scand.*, **13**, 1.
Goldenberg, M., Pines, K. L., Baldwin, E. F., Greene, D. G., and Roh, C. E. (1948). *Amer. J. med.*, **5**, 792.
Kroneberg, G., and Schümann, H. J. (1950). *Arch. exp. Path. Pharmak.*, **209**, 350.

Brit. J. Pharmacol. (1951), **6**, 289.

INSULIN AND THE SUPRARENAL GLAND OF THE RABBIT

BY

G. B. WEST

From the Pharmacological Laboratory, University of St. Andrews, Medical School, Dundee

(Received February 2, 1951)

When animals are injected with insulin in amounts sufficient to produce the symptoms of hypoglycaemia, a discharge of adrenaline and/or *nor*adrenaline from the suprarenal medulla occurs which helps to restore the normal blood sugar. The discharge does not occur if the splanchnic nerve is severed, nor does it occur when insulin is injected into the suprarenal artery. Burn, Hutcheon, and Parker (1950b) found that insulin treatment in rats resulted in a decline in the total activity of suprarenal gland extracts and in the percentage of adrenaline in them. They suggested that *nor*adrenaline is the precursor of adrenaline in the rat, and that after several hours' depletion of the medulla the supply of *nor*adrenaline is restored more rapidly than it can be methylated to form adrenaline. A similar finding has already been reported in the cat, after electrical stimulation of the splanchnic nerve (Bülbring and Burn, 1949a).

Only minute quantities of *nor*adrenaline are found in extracts of normal suprarenal glands of rabbits. When the plasma in the adrenal vein before and after stimulation of the splanchnic nerve is analysed, small quantities of *nor*adrenaline can be detected in samples collected soon after stimulation commences; continued stimulation results in its disappearance, only adrenaline then being detected (West, 1950). It seemed of interest, therefore, to investigate the content of the gland of the intact unanaesthetized animal under insulin treatment.

METHODS

For this study 66 rabbits of both sexes were used. As far as possible each set of results was obtained with one rabbit weighing under 1 kg. and one rabbit weighing 1.5–2.5 kg. The weights are important, as younger rabbits are more sensitive to insulin than are older ones. They were kept without food overnight. In the morning, blood from the ear vein was removed for blood-sugar determinations (Hagedorn and Jensen method), and then the injections were given. The doses of insulin were 2, 3.15, or 5 units per kg. subcutaneously; these were sufficient to produce symptoms of hypoglycaemia. With the higher dose levels symptoms were sometimes severe; these animals were either given intravenous injections of glucose or killed.

At a given time after the insulin injection, blood was again removed from the ear vein for sugar estimations. Immediately afterwards, the rabbits were killed by a blow on the head and bled out by cutting the carotid artery. The suprarenals were removed, dissected free from the capsules, weighed, and ground in a mortar with sand and 10 ml. 0.1 N–HCl/g. (Holton, 1949). The acid extracts were filtered, and the filtrates assayed for their adrenaline

and *nor*adrenaline contents by the action on the blood pressure and normal nictitating membrane of a spinal cat (Burn, Hutcheon, and Parker, 1950a). Total activity was usually determined in the isolated rabbit ileum. In certain experiments the extracts were assayed on the isolated rectum of a chick, the isolated uterus from a non-pregnant rat in dioestrus, or the uterus of a non-pregnant spinal cat. Assays were always completed within 24 hours from the time of killing the animals. Solutions of *l*-adrenaline and *l-nor*adrenaline were prepared in 0.01 N-HCl.

In a few experiments the glands were ground in a mortar with 0.25–0.5 ml. 0.1 N-HCl and then centrifuged. A measured volume of the clear supernatant was used for chromatographic identification and determination (James, 1948; Euler and Hamberg, 1949). An ascending column was used with a butanol–acetic acid–water solvent. For developing, 0.44 per cent potassium ferricyanide solution in phosphate buffer *p*H 7.8 was used. The remainder of the supernatant was diluted and used for the biological determination, as described above.

Results

Changes in total activity.—The changes in total activity for all experiments are shown in Table I. The mean control value of 485±131 μg. adrenaline per g. fresh tissue was obtained from 24 rabbits. There were slight variations in each group

TABLE I

THE INFLUENCE OF INSULIN ON THE ADRENALINE CONTENT OF THE SUPRARENAL GLANDS OF THE RABBIT

Dose of insulin (units/kg.)	Adrenaline content (μg./g.) at various times after insulin							
	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.	8 hr.	12 hr.
2.00	821	648	220	173	208	473	740	640
3.15	1,044*	922	355	170	189	450	735	750
5.00	1,117†	700	274‡	95‡	235‡	—	—	—
Mean ..	994	757	283	145	211	462	737	695

Control value of adrenaline in rabbit adrenals = 485 ± 131 μg./g.

* 50 μg. *nor*adrenaline/g. also found. † 41 μg. *nor*adrenaline/g. also found. ‡ I.V. glucose needed.

after insulin, and usually the value for the younger animal was lower than that recorded for the older one. During the first two hours the adrenaline content of the gland rose significantly, and it was only during this period that *nor*adrenaline was detected in certain extracts and estimated in amounts representing less than 6 per cent of the total amount. The adrenaline content dropped sharply during the next two hours, reaching maximum depletion at about four hours. From the biological tests there was no doubt at this stage that only adrenaline was present (see Fig. 1); a negative value was also obtained in the chromatographic experiments, indicating that less than 5 per cent *nor*adrenaline was present in the total mixture. By the sixth hour the values had reached the control level and only adrenaline was detected. The mean results are plotted in Curve A (Fig. 2). It will be noted that intravenous glucose was only necessary two hours after the high dose of insulin.

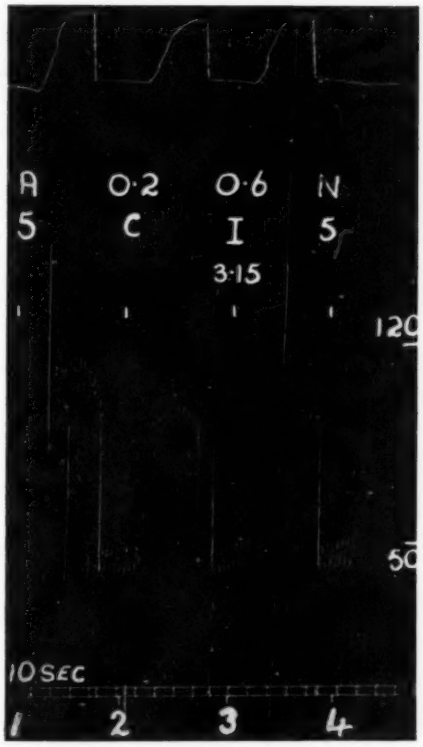


FIG. 1

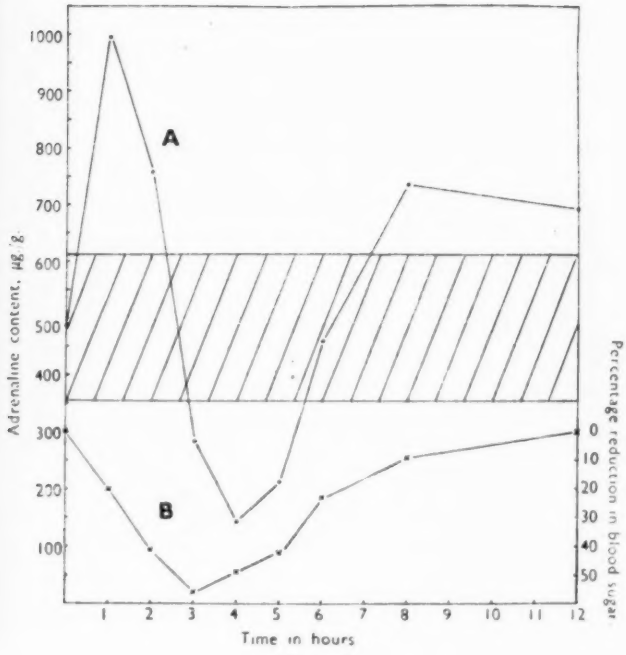


FIG. 2

FIG. 1.—Spinal cat. Normal nictitating membrane and blood pressure. (1) 5 µg. *l*-adrenaline; (2) 0.2 ml. extract of suprarenal glands of control rabbit; (3) 0.6 ml. extract of suprarenal glands of rabbit given 3.15 units insulin/kg. 4 hours previously; (4) 5 µg. *l-nor*adrenaline.

FIG. 2.—Curve A shows mean change in adrenaline content of rabbit suprarenals after insulin. Ordinates (on left) µg./g. Abscissa time in hours. Curve B shows mean percentage blood-sugar reduction (ordinate on right). Note the initial increase in adrenaline content in the first two hours. Values above or below the shaded area are significant.

Changes in blood sugar.—The percentage reduction in blood-sugar content was determined in each experiment, and the mean results for each group are shown in Table II. The maximum decline occurred at about three hours and recovery had almost taken place by eight hours (Fig. 2, Curve B).

TABLE II

THE EFFECT OF INSULIN ON THE BLOOD SUGAR OF RABBITS

Dose of insulin (units/kg.)	Percentage blood-sugar reduction at various times after insulin							
	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.	8 hr.	12 hr.
2.00	20	48	54	41	36	18	3	1
3.15	18	41	55	57	49	29	15	- 2
5.00	22	49	58	—	—	—	—	—
Mean ..	20	46	56	49	42	23	9	0

Discussion

The results show in the first place the change in total activity of the suprarenal medulla of the rabbit after injections of insulin in sufficient amounts to cause symptoms of hypoglycaemia. In the first two hours, while the blood sugar is steadily falling, a significant increase in activity occurs; the gland is calling on its reserves, which appear to be quite extensive. A similar increase in the total activity of the stimulated gland compared with that of the unstimulated gland of the opposite side was previously noted in the eviscerated animal under urethane anaesthesia (West, 1950) with the splanchnic nerve stimulated electrically for periods up to two hours. After the initial increase a rapid decline in total activity occurs, minimal values being recorded four hours after the insulin injection, approximately one hour after the maximal percentage blood-sugar reduction. At this stage the gland is being quickly exhausted in response to the hypoglycaemia, and total activity falls to less than 30 per cent of that originally present. By six hours recovery has taken place; in fact, the process of catechol formation appears to be proceeding at a fast rate, for total activity values obtained at eight and twelve hours are raised above the control value, despite the fact that the blood sugar is still below the normal fasting level (mean value 85 mg. per 100 ml.).

The results show in the second place the presence of small quantities of *nor*-adrenaline in the suprarenal gland about one hour after the injection of insulin. This is the only occasion when *nor*adrenaline has been detected. In the anaesthetized animal (West, 1950) it was shown that *nor*adrenaline was released into the plasma in the suprarenal vein only during the first ten minutes of continuous electrical stimulation of the splanchnic nerve, and that adrenaline was present in large quantities at all times.

Evidence for the assumption that *nor*adrenaline is the precursor of adrenaline has been obtained by Bülbring (1949), who showed that minced suprarenal tissue from cat and from dog was able to convert *nor*adrenaline to adrenaline, provided that adenosine triphosphate was present. Bülbring and Burn (1949b) also showed that *nor*adrenaline is converted into adrenaline in the isolated perfused suprarenal gland of the dog.

Under the influence of insulin (large or small doses) the suprarenal gland of the rabbit calls on its reserves and the output of adrenaline is increased, traces of *nor*-adrenaline then appearing. As the gland becomes exhausted, the work of replenishing the store of *nor*adrenaline in the medulla may proceed more slowly than the process of methylation, and only adrenaline is found. It may well be that *nor*adrenaline is not a precursor of adrenaline in the rabbit. This mechanism does not compare, therefore, with that seen in the cat, dog, or rat. In these animals prolonged stimulation results in a greater outpouring of *nor*adrenaline as time proceeds, so that there is a fall in total activity, and also a fall in the percentage of adrenaline, in the gland. Nevertheless, Burn, Hutcheon, and Parker (1950b) found in rats that two hours after the injection of insulin the total activity of the gland had decreased but the percentage of adrenaline in the mixture had risen slightly (indicating increased methylation). It is possible that a shorter time interval might have disclosed an increase in total activity similar to that seen in the rabbit.

Summary

1. When insulin is injected in sufficient amount the activity present in the suprarenal medulla of the rabbit first increases and then declines. The increase occurs during the first two hours after injection, and the fall is evident after three hours. The minimum value is reached at about four hours and recovery has taken place by six hours.

2. During the first two hours after injection of insulin, *nor*adrenaline in small amounts may appear in the extract of the suprarenal gland. At all other times the gland contains only adrenaline.

3, If *nor*adrenaline is a precursor of adrenaline in the suprarenal gland of the rabbit, then methylation to form adrenaline occurs rapidly, even in the exhausted gland.

I wish to record my thanks to Professor R. B. Hunter for his helpful advice, and to Mr. D. M. Shepherd of this Department for carrying out the chromatographic studies.

References

Bülbring, E. (1949). *Brit. J. Pharmacol.*, **4**, 234.
Bülbring, E., and Burn, J. H. (1949a). *Brit. J. Pharmacol.*, **4**, 202.
Bülbring, E., and Burn, J. H. (1949b). *Brit. J. Pharmacol.*, **4**, 245.
Burn, J. H., Hutcheon, D. E., and Parker, R. H. O. (1950a). *Brit. J. Pharmacol.*, **5**, 142.
Burn, J. H., Hutcheon, D. E., and Parker, R. H. O. (1950b). *Brit. J. Pharmacol.*, **5**, 417.
Euler, U. S. v., and Hamberg, U. (1949). *Nature, Lond.*, **163**, 642.
Holton, P. (1949). *J. Physiol.*, **108**, 525.
James, W. O. (1948). *Nature, Lond.*, **161**, 851.
West, G. B. (1950). *Brit. J. Pharmacol.*, **5**, 542.

Brit. J. Pharmacol. (1951), **6**, 294.

THE EFFECT OF ADRENALINE, *NOR*ADRENALINE, AND *ISO*PROPYL *NOR*ADRENALINE ON THE ASCORBIC ACID CONTENT OF THE RAT'S ADRENAL

BY

I. G. JARRETT

From the Department of Pharmacology, University of Edinburgh

(Received March 17, 1951)

Many of the observations that have followed the original work of Szent-Györgi on the ascorbic acid content of the adrenal gland have been directed towards a study of the significance of this vitamin in relation to the physiological state of the adrenal cortex. A wide variety of changes in environmental conditions are known to produce a depletion of the ascorbic acid content of the adrenal gland. The fall in the concentration of ascorbic acid in the adrenal has been used as a measure of enhanced adrenal cortical activity, and this enhancement has been presumed to be caused by the release of adrenocorticotrophic hormone from the anterior pituitary (Sayers, Sayers, Liang, and Long, 1945).

The injection of adrenaline has been shown by Vogt (1944) to produce a large increase in the amount of cortical hormone in the adrenal effluent of the dog, and Long and Fry (1945) observed a fall in the concentration of ascorbic acid in the adrenals of rats after the subcutaneous injection of this amine. Nasmyth (1949), in a study of the effect of some sympathomimetic amines, also reported a large depletion in the level of ascorbic acid in the adrenal of the rat after the subcutaneous injection of adrenaline, and a smaller depletion after that of *nor*adrenaline

In view of the increasing interest in substances which will release adrenocorticotrophic hormone (ACTH) when given in non-toxic doses it was decided to extend these studies, and particularly to compare the release of ACTH induced by *iso*propyl-*nor*adrenaline with that seen after adrenaline and *nor*adrenaline.

METHODS

Female rats of the Wistar strain (body weight 140–200 g.) were used for these investigations. The day before the experiment the animals were removed from the stock cages, weighed, marked, and then placed in a room thermostatically controlled at about 22° C. They were left undisturbed overnight and used with a minimum of handling on the day of the experiment.

The drugs were administered by subcutaneous injection, and after the required time interval had elapsed (1–4 hours) each animal was killed by a sharp blow on the head and then decapitated. The left adrenal gland was rapidly removed, carefully stripped of all extraneous tissue, and weighed to the nearest 0.1 mg. The gland was immediately immersed in a small volume of 4.5 per cent (w/v) trichloroacetic acid, homogenized, and the volume made up to 10 ml. with 4.5 per cent trichloroacetic acid. The ascorbic acid was estimated by the method of Roe and Kuether (1943), and the content of the gland was expressed as mg. ascorbic acid per 100 g. tissue.

Eighteen rats were demedullated bilaterally by enucleating the gland from the capsule and then allowing sufficient time to elapse for regeneration of cortical tissue. These animals were kept in the warm room for three weeks after the operation and thereafter subjected to normal room temperatures. During the first week they were given 0.9 per cent saline to drink. They were used about forty-five days after operation; good regeneration had taken place by this time.

The amines used were *l*-adrenaline, *l-nor*adrenaline *d*-bitartrate monohydrate, and *dl-iso*propyl-*nor*adrenaline hydrochloride (isoprenaline). A stock solution of each amine in dilute hydrochloric acid was made containing 0.1 g. of the base per 100 ml. and the *p*H adjusted to 3–4. Immediately before injection appropriate dilutions in physiological saline were made (usually 1/10 or 1/20) and the *p*H was adjusted to about 7 with sodium bicarbonate. The doses varied between 0.005 and 0.060 mg. base per 100 g. body weight.

In order to assess the extent to which the experimental procedure might have produced emotional excitement, with a consequent effect on the ascorbic acid content of the adrenals, a group of rats was injected with 0.9 per cent saline and killed at various times thereafter.

TABLE I

Ascorbic acid concentration (mg./100 g. fresh gland) in the adrenal gland after subcutaneous injection of saline, *l*-adrenaline, *l-nor*adrenaline, and isoprenaline into normal rats and into rats whose adrenals were demedullated. Control rats had an ascorbic acid content of 420±24 mg./100 g.

Drug	Dose mg./100 g.	Ascorbic acid (mean and S.E. of mean) at various times after injection. Number of rats in parentheses		
		1 hr.	2 hr.	3 hr.
0.9% saline	0.9	383±16(13)	359±9(10) 396±24(7)*	375±28(6)
l-adrenaline	0.01	283±7(6)	258±17(9) 279±17(6)*	300±16(10)
	0 02	262±11(5)	230±16(4) 354±14(8)	250±8(5)
*l-nor*adrenaline ..	0.0075 0.015	319±17(6)	283±6(6)	340±21(4)
	0.030	290±20(3)	283±6(9)	275±8(5)
	0 060		278±13(8)	287±8(4)
isoprenaline	0.005	269±12(13)	300±9(10)	292±16(7)
	0.010	267±17(6)	243±11(13)	300±11(6)
	0.015	271±12(11)	257±14(10) 260±17(5)*	288±15(7)
	0.020	249±14(6)	213±16(6)	296±12(6)
	0.030	234±4(3)	224±15(6)	234±16(5)

* Rats whose adrenals had been demedullated 45 days previously.

RESULTS

A series of animals which received no injection but were otherwise kept under the same environmental conditions as the injected groups had an average concentration of ascorbic acid in the adrenal gland of 420 mg./100 g. (S.E. of mean ±24). It will be seen from Table I that the injection of saline provided a sufficient stimulus to cause some depletion of the ascorbic acid. The minimum figure determined was at the two-hour period. This reduction in the level of the ascorbic acid was small,

however, when compared with that produced by the various amines (see Fig. 1). After four hours the values had usually returned to the pre-injection level.

l-Adrenaline in doses of 10 μg. and 20 μg. per 100 g. led to a large depletion. After *l-nor*adrenaline the depletion was less pronounced, and a dose as high as

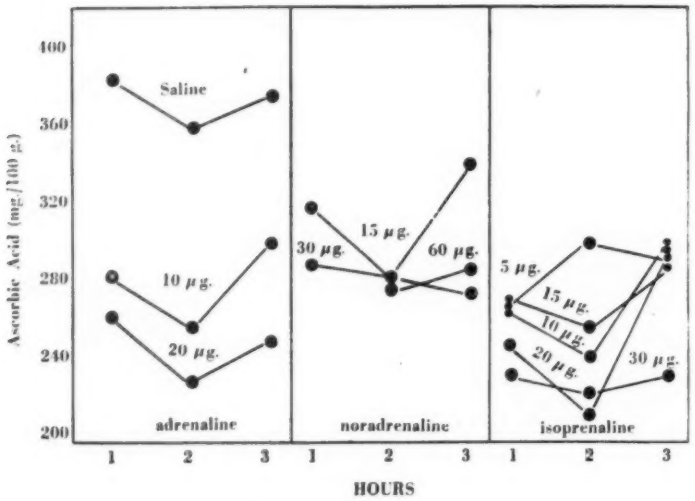
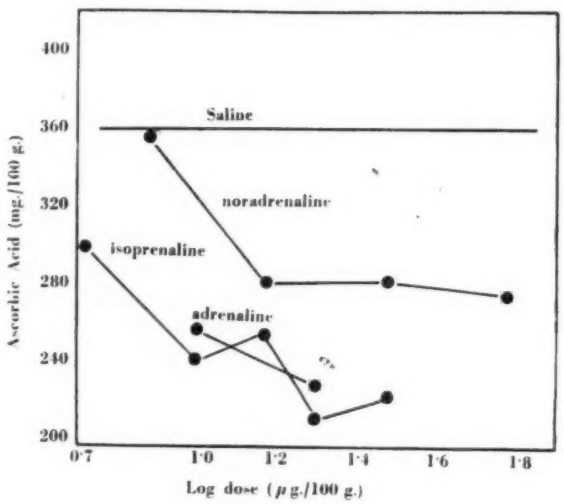


FIG. 1.—Concentrations of ascorbic acid (mg./100 g.) in rats' adrenal glands after subcutaneous injection of saline, adrenaline, *nor*adrenaline, and isoprenaline plotted against time in hours.

60 μg. per 100 g. failed to produce a response equivalent to that obtained with much smaller amounts of adrenaline. Isoprenaline, however, in spite of being injected as a racemic mixture of which the *d*-isomer is probably inactive, produced effects as large as did equal doses of *l*-adrenaline.

FIG. 2.—Adrenal ascorbic acid (mg./100 g. tissue) two hours after injection of saline, adrenaline, *nor*adrenaline, and isoprenaline plotted against log dosage.



The effect was fully developed two hours after the injection, except that the smallest dose of isoprenaline had its maximum effect earlier. The values determined two hours after injection were used for plotting a dose-response curve for each drug.

These dose-response curves (Fig. 2) reveal large differences in potency between *l-nor*adrenaline on the one hand and *l*-adrenaline and isoprenaline on the other. Comparable effects were obtained with 10 μg. *l*-adrenaline and 10 or 15 μg. isoprenaline, and also with 20 μg. *l*-adrenaline and 20 or 30 μg. isoprenaline. As isoprenaline was used in the racemic form, it would appear that it is at least as active as adrenaline in producing depletion of the ascorbic acid in the rat's adrenal. *Nor*adrenaline was less effective, and its efficacy did not increase significantly with the larger doses (up to 60 μg./100 g.).

In order to determine whether the high activity of isoprenaline was a property of the amine itself or due to the liberation of endogenous adrenaline, experiments were carried out on demedullated rats. The results obtained are marked with an asterisk in Table I. Two hours after a saline injection the ascorbic acid level in the demedullated group was slightly, though not significantly, higher than in the normal unoperated group. After injection of adrenaline and isoprenaline, however, the fall in ascorbic acid was virtually the same in demedullated and in normal rats.

Discussion

Equal doses of *l*-adrenaline and *dl-iso*propyl-*nor*adrenaline (isoprenaline) produced comparable falls in the ascorbic acid concentration of rats' adrenals. On the assumption that the *dextro*-isomer of isoprenaline is pharmacologically inactive, it would appear that the *laevo*-compound is more potent than *l*-adrenaline in depleting adrenal ascorbic acid, or, if we accept the usual interpretation of this depletion, in releasing ACTH. In confirmation of Nasmyth's work (1949), *l-nor*adrenaline was found much less potent than *l*-adrenaline. The fact that a fourfold increase in the dose of *nor*adrenaline was not associated with an appreciable increase in the loss of ascorbic acid may be connected with the observation that *l-nor*adrenaline, in spite of its greater pressor effect, is less toxic than *l*-adrenaline (Tainter, Tullar, and Luduena, 1948). In the anaesthetized dog, Vogt (unpublished) was unable to obtain proof of enhanced adrenocortical secretion after infusion of doses of *l-nor*-adrenaline which were much larger than the amounts of adrenaline required to accelerate cortical secretion. The lack of correlation between pressor effect and action on the hypophysial-adrenal system is even more striking when *nor*adrenaline is being compared with isoprenaline, since the latter drug lowers the blood pressure. It is because of this depressor effect that the experiments on isoprenaline were repeated in rats with demedullated adrenals, as it was conceivable that the fall in blood pressure which follows injection of isoprenaline might give rise to a compensatory secretion of adrenaline with consequent release of adrenocorticotrophic hormone. However, as the same fall in adrenal ascorbic acid was obtained after isoprenaline in normal and in demedullated rats, it would appear that any effect due to release of endogenous adrenaline was insignificant compared with the direct effect of the isoprenaline.

Ahlquist (1948), in a study of receptors for sympathomimetic amines, attempted to classify the effects of amines of this type on different physiological functions and found some tissue responses highly sensitive to *nor*adrenaline and little sensitive to isoprenaline, whereas the reverse was true for other responses. He postulated two types of cell receptors to account for these differences, calling the first type α and the

second β receptors. According to this classification it would be possible to consider the hypothetical receptors responsible for release of adrenocorticotrophic hormone as belonging to the β class.

From the results of the saline injections it is apparent that even under conditions of minimum handling there was some depletion of adrenal ascorbic acid; this stresses the necessity of careful control of all manipulations in experiments which measure the release of ACTH in conscious animals.

Summary

1. The concentration of ascorbic acid in the adrenal gland of the rat was determined after the subcutaneous injection of *l*-adrenaline, *l-nor*adrenaline, and *dl-iso*propyl-*nor*adrenaline (isoprenaline).

2. A large depletion was observed two hours after the injection of adrenaline and a much smaller depletion after that of *nor*adrenaline.

3. Isoprenaline is as potent as *l*-adrenaline in depleting ascorbic acid in the adrenal gland, in spite of the fact that it consists of two isomers, one of which may be inactive.

4. The depletion of ascorbic acid after the injection of adrenaline and isoprenaline is not diminished by adrenal demedullation. The effect of the injection of isoprenaline cannot therefore be explained by the release of endogenous adrenaline.

This work was done while the author was on a visit from the Division of Biochemistry and General Nutrition of the Commonwealth Scientific and Industrial Research Organization, University of Adelaide, South Australia. The author also wishes to acknowledge the valuable help and interest of Dr. Marthe Vogt under whose suggestion and guidance this work was carried out.

References

Ahlquist, R. P. (1948). *Amer. J. Physiol.*, **153**, 586.
Long, C. N. H., and Fry, E. G. (1945). *Proc. Soc. exp. Biol., N.Y.*, **59**, 67.
Nasmyth, P. A. (1949). *J. Physiol.*, **110**, 294.
Roe, J. H., and Kuether, C. A. (1943). *J. biol. Chem.*, **147**, 399.
Sayers, G., Sayers, M., Liang, T.-Y., and Long, C. N. H. (1945). *Endocrinology*, **37**, 96.
Tainter, M. L., Tullar, B. F., and Luduena, F. P. (1948). *Science*, **107**, 39.
Vogt, M. (1944). *J. Physiol.*, **103**, 317.

Brit. J. Pharmacol. (1951), **6**, 299.

THE PHARMACOLOGY OF THE TOXIFERINES

BY

W. D. M. PATON AND W. L. M. PERRY

From the National Institute for Medical Research, Mill Hill, London, N.W.7

(Received March 3, 1951)

We owe to Boehm (1895) the classification of South American arrow poisons into tube, pot, and gourd (or calabash) curare—a classification based primarily on the type of container in which the curare was found. Since that time much botanical and chemical effort has been expended in attempts to distinguish and isolate the active principles of these different curares (see MacIntyre, 1947). The success of King (1935) in isolating and characterizing from tube-curare the crystalline alkaloid *d*-tubocurarine chloride has resulted in the widespread use of this particular alkaloid, or of preparations containing it, and the consequent amassing of detailed knowledge about its pharmacological actions. The pharmacological studies of other alkaloids have been directed largely to establishing no more than their potency, particularly because the most active alkaloids, those from calabash curare, have proved very difficult to isolate in quantities sufficient for pharmacological study. Thus Wieland, Bähr, and Witkop (1941) were only able to prepare a few micrograms of the materials which they named Toxiferines I and II.

Recently King (1949) has isolated, from the bark of *Strychnos toxifera*, which is known to be one of the principal ingredients of calabash curare, somewhat larger quantities to these two toxiferines, together with ten other substances, and we are indebted to him for supplying us with samples of eight of these alkaloids. The opportunity has thus arisen to study in some detail the properties of these substances and to compare them with *d*-tubocurarine. Toxiferines I and II were extracted as chlorides, the remaining ten as picrates, from 5 kg. bark. There were available for pharmacological test six of these picrates (Toxiferines IV, V, VI, IX, XI, and XII), about 3 mg. of each, and a somewhat more abundant supply of the two chlorides (Toxiferines I and II) amounting to 10 mg. of each salt.

Our experiments were intended to discover the main properties of the compounds and to analyse the type of block produced at the neuromuscular junction and at the ganglionic synapse, in view of the differences already described in the modes of action of other myoneural and ganglionic blocking agents (Burns and Paton, 1950; Paton and Perry, 1951).

METHODS

The methods used throughout were standard, and most of them were described briefly by Paton and Zaimis (1949); endplate potentials were recorded by the method of Brown and Burns (1949), and ganglion potentials as described by Paton and Perry (1951).

RESULTS

Neuromuscular block

Effect on righting reaction of the frog.—The most rapid and economical method of assessing the potencies of the toxiferines was the classical one of determining their effect on the righting reaction of the frog. This has the additional advantage that it was the method used by Wieland, Bähr, and Witkop (1941) for toxiferines I and II. Our results with this method are summarized in Table I. Three sets of experiments

TABLE I

EFFECT OF TOXIFERINES ON THE RIGHTING REACTION OF FROGS

Toxiferine No.	March		August		October	
	ED50 μg./kg.	Potency × *d*.t.c.	ED50 μg./kg.	Potency × *d*.t.c.	ED50 μg./kg.	Potency × *d*.t.c.
I (chloride)	7.5	240			14	76
VI (picrate)			15	170	15	61
XI ,, ...			15	170	27	38
IV ,, ...			100	30		
V ,, ..			250	10		
IX ,, ...			400	6		
II (chloride)	4,600	0.4				
XII (picrate)			>1,160	<2		
d-tubocurarine chloride ..	1,800	—	2,500	—	900	—

were done, in spring, summer, and autumn; at each season the potency of *d*-tubocurarine was also determined. A sufficient number of animals was available during the spring and autumn assays to permit of a legitimate probit analysis of the results, and the figures for potency in terms of *d*-tubocurarine for these assays have been calculated from this analysis. In the summer assays, estimates of the ED50's were made by graphical interpolation, and the potency ratios with respect to *d*-tubocurarine were calculated directly.

The striking feature of these tests was the outstanding potency of three of the toxiferines, namely, Nos. I, VI, and XI, which were active at all seasons of the year in doses of less than 30 μg./kg. In the March estimate of the potency of toxiferine I, the ED50 was only 7.5 μg./kg., so that the dose to paralyse a 20 g. frog was then as low as 150 mμg. These figures for toxiferine I agree well with the rough figure of 300 mμg. for frogs of unspecified weight given by Wieland *et al.* (1941), and confirm that this compound and toxiferines VI and XI are the most potent known curarizing agents.

Of the remaining toxiferines, three are more active than *d*-tubocurarine (Nos. IV, V, IX), and the other two which we have tested are probably less potent.

Apart from differences in potency, the paralysis of a frog by toxiferines and by *d*-tubocurarine was much alike. With small doses, the animal was simply weakened, and the righting reaction was slowed but not abolished. Larger doses caused abolition of this reaction, but the animal was still capable of weak spontaneous movements and respiratory movements were still visible. A further increase in dose abolished all movements, respiratory or otherwise, although the heart could still be

seen beating through the thoracic wall. The whole gamut from the beginning of muscular weakness to complete paralysis was covered roughly by a 100 per cent increase in dose, both with the toxiferines and with *d*-tubocurarine. There was a definite difference in the duration of paralysis; the paralysis by *d*-tubocurarine was more rapid in onset and passed off more quickly than that due to the three most potent toxiferines.

We also found (Table I) that, whereas the potency of the toxiferines remains remarkably constant at different times of year, the absolute potency of *d*-tubocurarine varies considerably. It is this latter factor which is mainly responsible for the large swings in the relative potency of the toxiferines with respect to *d*-tubocurarine which were observed in the different assays.

It is known that there are many physiological differences between summer and winter frogs which, no doubt, account for the seasonal variations in the potency of *d*-tubocurarine. The relative constancy of the ED50's of the toxiferines for frogs at all seasons may be related to their remarkable constancy of potency in different species, described later.

Potency in other species.—In view of the very high potency of toxiferine I in the frog, together with the fact that our supply of this compound was relatively abundant, it was possible to extend our observations on its potency to other animal species. Table II summarizes the results of these experiments. The most striking

TABLE II

ACTIVITY OF TOXIFERINE I IN DIFFERENT SPECIES

Species	ED50 μg./kg.	Potency × *d*.t.c.
Frog (March)	7.5	240
Frog (October)	14	76
Mouse (righting reflex)	10	12
Rabbit (head-drop)	11	20
Cat (tibialis twitch)	15–20	15

fact about these observations is the similarity in the effective doses of toxiferine I, whether tested in frog, mouse, rabbit, or cat, the range of variation being no more than that among frogs at different seasons. The difference between the effective doses of *d*-tubocurarine for mammals and frogs presents a marked contrast.

In all species investigated, as in the frog, the onset of paralysis is slower and the duration of paralysis longer with the toxiferines than with *d*-tubocurarine. For instance, in the cat after an intravenous dose, the presence of toxiferine I is demonstrable two hours, or sometimes even three hours, later by the increased sensitivity of the animal to a further dose; and the period of actual paralysis is correspondingly longer than with *d*-tubocurarine.

Mechanism of muscular paralysis.—Although there could be little doubt that toxiferines, being the active principles of calabash curare, exerted their paralysing action by blocking transmission at the myoneural synapse, we thought it important to establish this for certain. To do this, it was necessary to show that the toxiferines affected neither muscle nor nerve directly. We found that there was no effect on

muscle, by observing that the response to direct stimulation of a cat's tibialis, fully paralysed to stimulation through its nerve, was completely unaffected. The usual way to demonstrate that a paralysis is not due to interference with conduction in the motor nerve is to record normal action potentials from the trunk of the nerve. The recording of an endplate potential, however, from a muscle paralysed with toxiferine, serves the same purpose. When the nerve impulse is blocked there is naturally no endplate potential; if, on the other hand, the nerve impulse is not blocked, a normal propagated response of the muscle fibre is elicited and the endplate potential is obscured by a spike potential. In neither case, therefore, can pure endplate potentials be recorded. Their occurrence, therefore, on nerve stimulation during motor paralysis is proof of block at the myoneural synapse. During paralysis after toxiferine I such pure endplate potentials can in fact be recorded, and Fig. 1 shows two endplate potentials recorded from the endplate region of a cat's gracilis after a dose of 200 μg. toxiferine I intravenously; the upper record is with complete paralysis, and the lower record was taken during partial recovery and shows the beginning of propagated spikes.

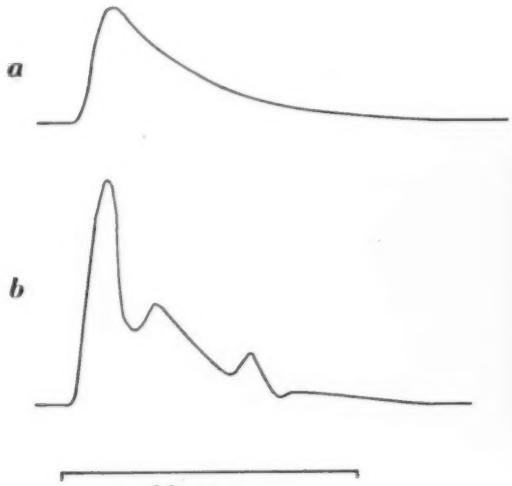


FIG. 1.—Cat, chloralose, gracilis. Records of endplate potentials. Electrodes on musculo-tendinous junction and endplate region. Intravenous injections. (*a*) After toxiferine I, 200 μg.; (*b*) later, during recovery from same dose of toxiferine I.

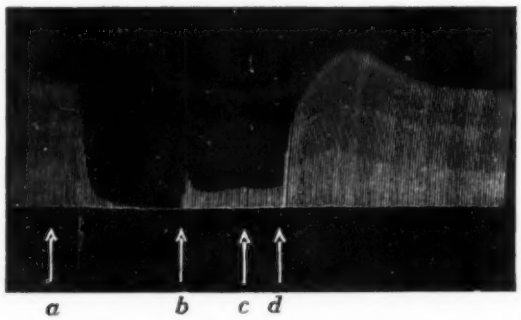


FIG. 2.—Cat, chloralose. Record of contractions of tibialis excited by supramaximal shocks to the sciatic nerve every 10 seconds. Intravenous injections. At (*a*) toxiferine VI, 20 μg./kg.; at (*b*) tetanus, 25 shocks per sec. for 10 sec.; at (*c*) atropine sulphate, 0.3 mg./kg.; and at (*d*) prostigmine methylsulphate, 0.3 mg./kg.

Characteristics of neuromuscular block by toxiferines.—Several characteristic features of toxiferine-induced block of the cat's tibialis to nerve stimulation are shown in Fig. 2; 20 μg./kg. of toxiferine VI causes, after a latency of about one minute, a smooth and rapid onset of block. There is no preceding potentiation of the tension of the single twitch, nor any sign of muscular fasciculations; both these phenomena are typical of block by decamethonium and are absent with *d*-tubocurarine. Atropine causes a slight intensification of the effect of toxiferine VI. Prostigmine, given after the atropine, reverses the block very rapidly and even

produces a distinct potentiation of the maximal twitch tension. This antagonistic action of prostigmine was also shown for block due to toxiferine I and toxiferine XI.

When a brief tetanus of 25 shocks per second for 10 seconds is applied to the motor nerve at the height of the block (Fig. 2 (*b*)), the contraction of the muscle is well sustained, and the tetanus is followed by a definite reversal of the block to single shocks. The same decurarization after a tetanus was observed after toxiferine I and toxiferine XI. But the degree to which a tetanus was held, during block by the latter two toxiferines, was usually considerably less than with toxiferine VI. We also noted that there was considerable variation in the degree to which a tetanus was sustained, not only after doses of different toxiferines, but also after repeated doses of the same toxiferine.

It is only in this last respect that the toxiferines differ from *d*-tubocurarine or resemble decamethonium: for a nerve-excited tetanus is always poorly held with the former and well maintained with the latter. In the absence of an initial stimulant phase, the effectiveness of prostigmine, and the decurarization by a tetanus, the properties of the toxiferines are qualitatively identical with those of *d*-tubocurarine and dissimilar from those of decamethonium.

Antagonism to acetylcholine on frog's rectus.—One of the characteristic effects of *d*-tubocurarine is its antagonism to the action of acetylcholine on the frog's rectus

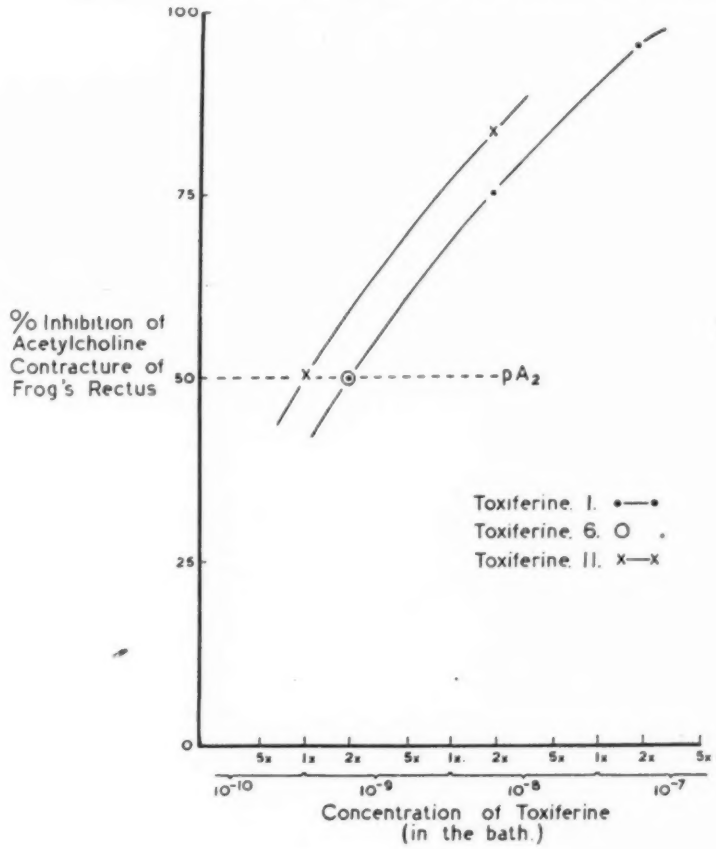


FIG. 3.—Frog's rectus abdominis. Graph of the antagonism by toxiferines to contracture produced by acetylcholine.

preparation: decamethonium, on the other hand, potentiates the effects of acetylcholine on this preparation, and itself produces a contracture. This experiment provides a simple and crucial test for analysing the nature of the neuromuscular block produced by toxiferines. The addition of a few micrograms of a toxiferine to the bath containing the frog rectus preparation produced a definite antagonism to acetylcholine, and there was no sign that the toxiferine itself could cause a contracture. The antagonism was measured quantitatively as follows: Several contractions to the same dose of acetylcholine were recorded; then toxiferine was added to the bath, and this reduced the acetylcholine response; the amount of acetylcholine necessary, in the presence of the toxiferine, to restore the response to the original size was then determined. Thus, if the original dose of acetylcholine must be trebled during toxiferine paralysis in order to produce the original effect, then two-thirds of this acetylcholine have been antagonized; i.e., there is 67 per cent inhibition. Fig. 3 gives the dosage-response lines relating the concentration of toxiferine in the bath with the percentage inhibition of acetylcholine calculated on this basis, and shows that toxiferine XI is more potent than toxiferines I and VI.

pA_2 (Schild, 1947) is the negative logarithm of the concentration of an antagonist which causes 50 per cent inhibition of some effect, and we have indicated it by a dotted line in Fig. 3. The single point determined for toxiferine VI corresponds exactly with a similar point for toxiferine I. The pA_2 for all three compounds thus lies between 8 and 9. This value is comparable with that of atropine against the action of acetylcholine on the guinea-pig's ileum (8.27; Schild, 1947) and illustrates quantitatively their extremely high activity.

Effect on respiratory muscles.—The respiratory muscles have a particularly high sensitivity to the toxiferines. Fig. 4 compares the effect of toxiferine I on the cat's tibialis with its effect on the minute volume of the same cat. A dose of 9 μg./kg. of toxiferine I produced a depression of the minute volume of some 5–10 per cent, without in any way affecting the tibialis twitch. The dose was then increased by the injection of 14 μg./kg., which, allowing for partial elimination of the previous dose,

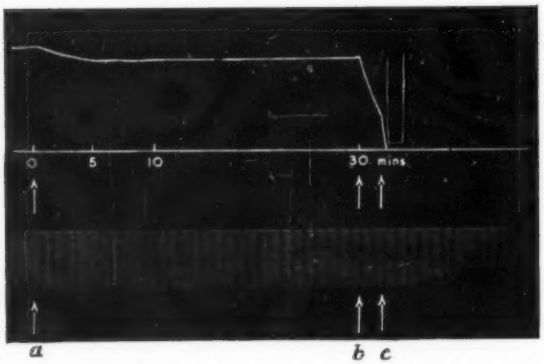


FIG. 4.—Cat, chloralose. Lower record, tibialis; nerve shocks every 10 sec. Upper record, graph of respiration. Intravenous injections. At (*a*) toxiferine I, 9 μg./kg.; at (*b*) toxiferine I, 14 μg./kg.; and at (*c*) artificial respiration started.

increased the total dose to about 20 μg./kg., with the result that the respiratory movements were depressed to such an extent as to require the application of artificial respiration; the blocking of the tibialis muscle was of the order of 40 per cent only. The original 5–10 per cent effect on the respiration was accompanied by a rise in blood-pressure, presumably asphyxial in origin. Similar results were obtained with

toxiferine VI, but not with toxiferine XI, which had no selective action on the respiratory muscles.

Endplate potentials in the muscle blocked by toxiferines.—The analysis of the neuromuscular block by toxiferines was taken a step further by means of experiments on the cat's gracilis muscle, in which the convenient localization of the endplates makes the recording of potentials from them a simple matter. Fig. 1 shows a record of such an endplate potential, after an intravenous dose of 200 μg. toxiferine I. Its time-course is similar to that of the endplate potential produced in a muscle treated with *d*-tubocurarine.

The size of the endplate potential depends on the rate of excitation of the motor nerve, in the same way as does the tension of the motor twitch. If paired shocks are applied with different intervals between them (Fig. 5), the second endplate potential is reduced in size by slightly less than 10 per cent for intervals between 20 and 150 msec. With a shorter interval, summation of the two endplate

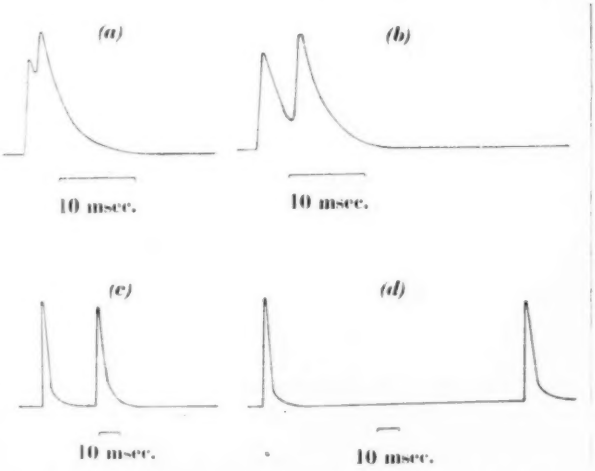


FIG. 5.—Cat, chloralose, gracilis. Records of endplate potentials after 75 μg. toxiferine I per kg. intravenously. Effect of paired shocks with different stimulus intervals: (*a*) 1.3 msec.; (*b*) 4.73 msec.; (*c*) 23.2 msec.; (*d*) 110 msec.

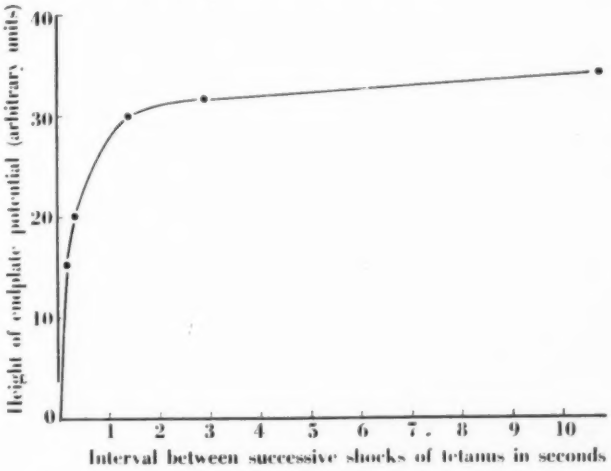


FIG. 6.—Cat, chloralose, gracilis. Graph relating the final height of the endplate potential during tetanic stimulation to the frequency of stimulation.

potentials occurs; but a correction can be made for the altered baseline of the second potential. It is then found that this depression in size of the second potential passes off gradually, as the interval is shortened, until the two potentials have the same height at an interval of about 5 msec. As the interval between the shocks is lessened still further, the second potential enters the refractory period of the first and begins to decay again.

When, instead of paired shocks, a tetanus is applied, the endplate potential falls continuously in magnitude until, in a second or two, it reaches a fairly constant level. This level varies with the rate of stimulation, and Fig. 6 is a graph of the relationship in one experiment between the final height of the endplate potential and the interval between the shocks of the tetanus. These results are obviously

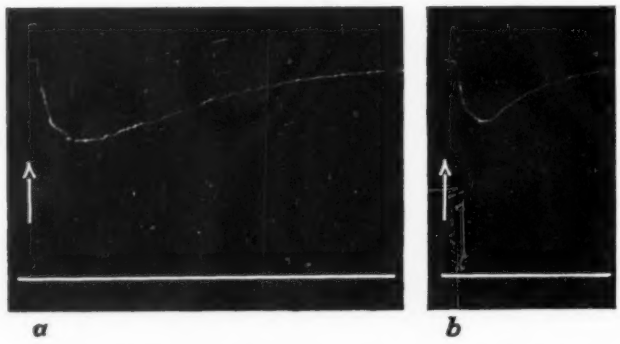
relevant to the behaviour of the partially paralysed muscle during a tetanus, but we have not sufficient information to characterize the relationship further.

Immediately after such a tetanus, the endplate potential increases in size, often to such an extent that propagation of impulses is restored. During the succeeding few minutes, block slowly returns. This post-tetanic augmentation of the endplate potential is obviously the basis of the increase in twitch tension after a tetanus, described earlier in this paper. The magnitude of this post-tetanic effect appeared to be greater with the toxiferines than with *d*-tubocurarine.

Ganglionic block

Effect on the cat's superior cervical ganglion.—Doses of 100 μg. of toxiferine I, given by rapid injection into the lingual artery, cause no contraction of the nictitating membrane ; but when the nictitating membrane is in a state of contraction due to continued preganglionic stimulation of the cervical sympathetic, 50 μg. toxiferine I produce partial relaxation (Fig. 7). The degree of block is similar to that produced by 25 μg. *d*-tubocurarine, although the duration is much longer.

Fig. 8 is a record of action potentials obtained from the cat's superior cervical ganglion. The recording electrodes were placed on the ganglion and the cut post-



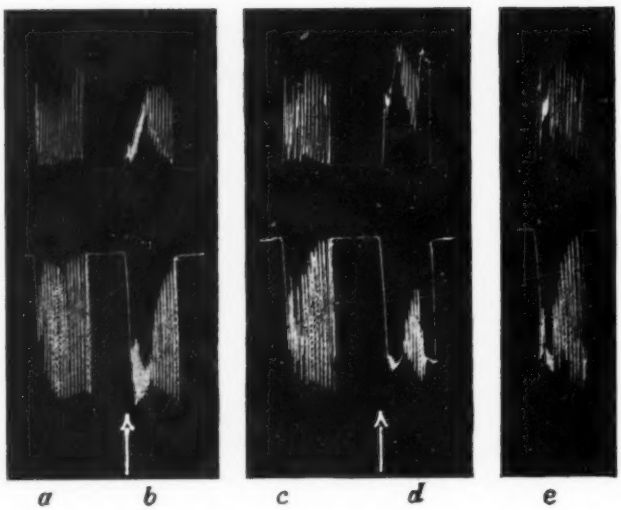


FIG. 7.—*Upper record :* Cat, chloralose, nictitating membrane stimulated preganglionically with supramaximal shocks at a rate of 10 per sec. Intra-arterial injections into lingual artery. At (*a*) 50 μg. toxiferine I; and at (*b*) 25 μg. *d*-tubocurarine. *Lower record:* Rabbit's ileum. Records of contraction of longitudinal muscle (top trace) and of volume change (bottom trace) in response to rise in pressure within lumen of gut. Normal controls (*a*), (*c*), and (*e*); before (*b*) 0.2 mg. *d*-tubocurarine and before (*d*) 0.1 mg. toxiferine I added to bath.

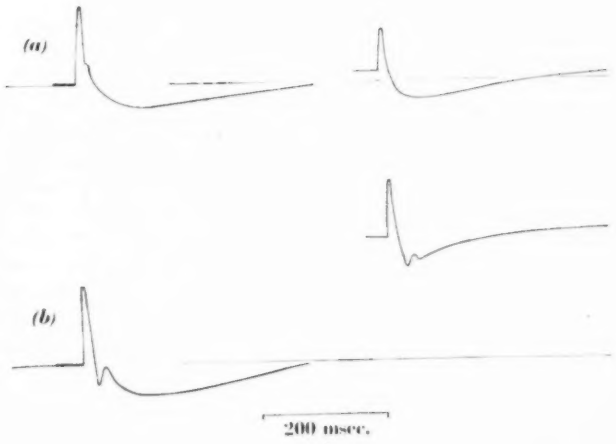


FIG. 8.—Cat, chloralose, superior cervical ganglion. Recording electrodes on ganglion and cut postganglionic trunk. Preganglionic stimulation. Intra-arterial injections into external carotid artery. (*a*) Before and after toxiferine I, 25 μg. (*b*) Before and after nicotine, 100 μg.

ganglionic trunk, the stimulating electrodes on the preganglionic trunk (Paton and Perry, 1951). The record (Fig. 8*a*) shows that the synaptic block by toxiferine is unaccompanied by a depolarization of the ganglion cells. This is to be compared with the records obtained with *d*-tubocurarine and with nicotine; *d*-tubocurarine block is also unaccompanied by depolarization, but after nicotine (Fig. 8*b*) there is a large depolarization of the ganglion cells accompanying the block. The mode of ganglionic blocking activity of the toxiferines appears, therefore, to be similar to that of *d*-tubocurarine.

Effect on the cat's parasympathetic submaxillary ganglion.—In one experiment a dose of 50 μg. toxiferine I was given intravenously to a cat, and records taken of the nictitating membrane stimulated preganglionically, and the salivary gland stimulated through the chorda tympani. In this experiment this dose produced a barely detectable effect on the nictitating membrane, together with a definite slowing of the rate of salivary secretion. A similar result was obtained with *d*-tubocurarine. This is in agreement with the general observation (Paton and Perry, unpublished) that the parasympathetic ganglion cells which supply the salivary gland are more sensitive to ganglion blocking agents than are the sympathetic ganglion cells supplying the nictitating membrane.

Effect on the myenteric plexus of the rabbit's ileum.—Fig. 7 (lower record) shows the effect of toxiferine I on the peristaltic reflex in the rabbit's ileum, the technique of Trendelenburg (1917) being used. The peristaltic waves of contraction of the circular muscle layer, in response to an increase in pressure in the lumen for one minute, are partially depressed by 0.2 mg. *d*-tubocurarine and by 0.1 mg. toxiferine I. It will be seen that 0.1 mg. toxiferine I is more potent than 0.2 mg. *d*-tubocurarine. *d*-Tubocurarine and toxiferine I affect only the waves of contraction of the circular muscle, which are dependent on the integrity of the myentric plexus, but not the contraction of the longitudinal muscle which is a myogenic response to the increased pressure.

Summary of ganglionic blocking actions.—The foregoing results show that the relative ganglionic blocking of toxiferine I compared with that of *d*-tubocurarine

varies considerably between the several autonomic ganglia investigated. Rough estimates of potency are as follows:

	Potency of toxiferine relative to *d*-tubocurarine
Cat's nictitating membrane	*c.* 0.5
Cat's salivary gland	3–4
Rabbit's ileum	*c.* 2.0

Other actions

Muscarine-like and atropine-like activity.—Our information about muscarinic activity was gleaned from experiments done for other purposes. A dose of 15 μg. toxiferine I per kg., which is too small to block sympathetic ganglia, given intravenously in the cat had no action on heart rate or blood-pressure, apart from the effects resulting from asphyxia due to respiratory paralysis. Furthermore, in the experiments on the rabbit ileum, a dose of 0.1 mg. toxiferine I did not itself cause any contraction of the gut. It follows from these observations that the muscarine-like activity of toxiferine I is certainly less than 1/1,000th that of acetylcholine, and must be negligible compared to its myoneural blocking action.

One test was made to determine whether toxiferine I had, like *d*-tubocurarine, any atropine-like activity on the guinea-pig's ileum. On this preparation the contraction to acetylcholine is either wholly or mainly an effect on the muscle fibres (Feldberg, 1950). The fact that toxiferine I produced an inhibition of the acetylcholine response of about 50 per cent (Fig. 9) indicates that the drug has an atropine-like activity. For this effect the approximate value for pA_2 is 4.2. Toxiferine I has, therefore, less than 1/5,000th the activity of atropine itself.

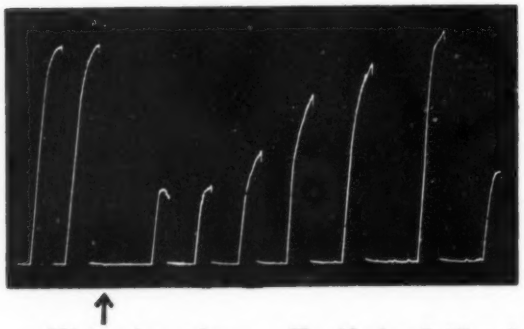


FIG. 9.—Guinea-pig's ileum. Records of contractions produced by 0.2 μg. acetylcholine, except for the final contraction which is the response to 0.1 μg. acetylcholine. At the arrow 0.1 mg. toxiferine I was added to the 15 c.c. bath, and remained in the bath only during the contraction immediately following.

Histamine release.—Toxiferine I, like *d*-tubocurarine, appears to cause the release of histamine, as shown by the following experiment. A solution of 1 mg./c.c. was injected in a volume of 0.02 c.c. intradermally into the skin of the forearm in two subjects, and a similar saline injection was made in an adjacent area. Five minutes after the injection there was a well-defined weal 5 mm. × 5 mm., with a flare 30 mm. × 25 mm. in each subject, with a sensation of itching at the sites of the toxiferine injection; at the control sites there was no more than the small lump formed by the injected fluid. It seems probable, therefore, that toxiferine I can liberate histamine from skin, although it is not a histamine-liberator of great potency; certainly its activity in this respect is small compared to its neuromuscular blocking power.

Discussion

In most respects the toxiferines share the pharmacological properties of *d*-tubocurarine. The paralysis is such that the muscle is always capable of a full contraction if excited directly, and such that a typical endplate potential can be recorded. The first observation is evidence that the muscle itself is not affected by the drugs. The second implies that the paralysis is not due to interference with nervous conduction ; for such a nerve-block could only progressively diminish the size of total propagated action potential at the endplate, and could not reveal the endplate potential itself. The two observations together constitute an unusual but valid way of demonstrating that toxiferine causes a true neuromuscular block.

Further common features to both drugs are the smooth onset of paralysis, antagonism by anticholinesterases, post-tetanic relief of block, and depression of the respiration. It follows from these findings that the paralysis must be due to a raising by the toxiferine of the threshold of the motor endplate to acetylcholine. We have not tested this directly, but the antagonism of toxiferine to acetylcholine on the frog's rectus abdominis verifies our conclusion on one type of skeletal muscle at least. It is clear, therefore, that the toxiferines must be classed with *d*-tubocurarine rather than with drugs such as decamethonium which cause neuromuscular block not by raising the endplate threshold to acetylcholine but by a prolonged depolarization of the endplate.

Nevertheless, there are certain important deviations from an over-all resemblance to *d*-tubocurarine which must be noted. In the first place, a muscle blocked with toxiferine often responds to a tetanus with a sustained contraction ; secondly, post-tetanic potentiation seems to be greater in the muscle exposed to toxiferine than in the muscle exposed to *d*-tubocurarine ; and thirdly, as Fig. 2 shows, prostigmine not only reverses block due to toxiferine, but also potentiates the normal twitch tension. This potentiation is not found in muscles blocked with *d*-tubocurarine.

In their actions on other organs, toxiferines show a qualitative resemblance to *d*-tubocurarine ; both produce a triple response in human skin, and have negligible muscarinic actions and a small but detectable atropine action. Representative members of the series can produce ganglion block ; and, on the superior cervical ganglion of the cat, at least, the block is of a similar type to that produced by *d*-tubocurarine, in that it occurs in the absence of any depolarization of the ganglion cells. The ganglionic activity of toxiferine is, however, of lower intensity than the neuromuscular action for, whereas it is 15 times as active as *d*-tubocurarine on muscle, it is less than 4 times as active as *d*-tubocurarine on all three ganglia tested in the cat and the rabbit. This provides yet another instance of the lack of correlation between the intensity of block at the myoneural and that at the ganglionic synapse. Since transmission at both synapses is mediated by acetylcholine, such discrepancies deserve closer examination.

The salient features of the pharmacology of the potent toxiferines are their high activity, and the uniformity with which that activity is displayed in different species. Weight for weight, they are more active than any other neuromuscular blocking drug, judged by any test. Since a substantial part of the molecule is anion, and since the toxiferine itself has certainly a high molecular weight, its molar potency is probably higher still, relative to other drugs. Certain other compounds,

indeed, have an activity of the same order in some species ; decamethonium iodide is active at 30 μg./kg. in the cat ; the dimethyl ether of *d*-tubocurarine at 10 μg./kg. in the rabbit. But no other compound displays so uniformly high an activity, not only in cat and rabbit, but in mouse and frog as well. Among the bewildering species differences found in the sensitivity to drugs of this type, importance must attach to a compound so exceptional in potency and uniformity of action. Given such a compound, stories frequent in the more adventurous traveller's tale of inexorable death after the merest scratch by a poisoned arrow take on some semblance of credibility.

References

Boehm, R. (1895). *Abh. sächs. Ges. (Akad.) Wiss.*, **22**, 203.
Brown, G. L., and Burns, B. D. (1949). *J. Physiol.*, **108**, 54*P*.
Burns, B. D., and Paton, W. D. M. (1950). *Proc. XVIII Int. Physiol. Congress*, 136.
Feldberg, W. (1950). *Proc. Roy. Soc. B*, **137**, 285.
King, H. (1935). *J. chem. Soc.*, 1381.
King, H. (1949). *J. chem. Soc.*, 3263.
McIntyre, A. R. (1947). *Curare*, University of Chicago Press, Chicago.
Paton, W. D. M., and Perry, W. L. M. (1951). *J. Physiol.*, **112**, 48*P*.
Paton, W. D. M., and Zaimis, E. J. (1949). *Brit. J. Pharmacol.*, **4**, 381.
Schild, H. O. (1947). *Brit. J. Pharmacol.*, **2**, 189.
Trendelenburg, P. (1917). *Arch. exp. Path. Pharmak.*, **81**, 55.
Wieland, H., Bähr, I., and Witkop, B. (1941). *Annalen*, **547**, 156.

Brit. J. Pharmacol. (1951), **6**, 311.

REVERSAL OF NICOTINE ACTION ON THE INTESTINE BY ATROPINE

BY

N. AMBACHE AND J. EDWARDS

From the Medical Research Council, Ophthalmological Research Unit, Institute of Ophthalmology, Judd Street, London, W.C.1

(Received March 2, 1951)

Evidence for the existence of an intrinsic inhibitory innervation in the wall of the intestine has been presented previously (Ambache, 1951). From experiments with botulinum toxin it could be deduced that the postganglionic neurones in the myenteric plexus are of two sorts, one set being motor and probably cholinergic, the other inhibitory and perhaps adrenergic (see Fig. 12 of the earlier paper). Botulinum toxin reversed the response to nicotine by producing a selective paralysis of the motor neurones, which allowed the effect of nicotine-stimulation of the inhibitory ganglia to be revealed.

Although the actions of atropine and of botulinum toxin at cholinergic nerve-endings are entirely different, yet, since atropine can also discriminate between cholinergic- and adrenergic-fibre effects, it was thought that reversal of nicotine action should be obtainable with atropine as with botulinum toxin. In preliminary experiments on rabbit's intestine, which are reported at the end of this paper, this expectation was not fulfilled. However, it was clear from these experiments that reversal could not be detected because of the persistence in the rabbit gut of the motor response to nicotine even after such high concentrations of atropine as 10^{-4}. But in preparations of intestine and of the gastric muscle from kittens it was possible to eliminate the motor response to nicotine with concentrations of atropine as low as 10^{-7} and to detect the occurrence of nicotine-reversal under conditions similar to those in which McSwiney and Robson (1929) observed "vagal reversal." An analogy between these two reversals produced by atropine is drawn in the discussion.

METHODS

Three adult rabbits (2.1–3.2 kg.) and six kittens, 8–12 weeks old (0.3–0.82 kg.), were killed by concussion. A segment of ileum was excised and suspended at once, with the lumen empty but closed at either end, in a 10-c.c. organ-bath full of Mg-free Tyrode (1.1 per cent $NaHCO_3$) oxygenated with a gas mixture of 95 per cent O_2 and 5 per cent CO_2.

The stomach strips were taken from two other 10-week kittens, both 0.49 kg. The stomach was removed and freed from omentum; after its contents had been washed out, the organ was slit open longitudinally, and the stomach wall was pinned down on a cork board with the mucosa facing upwards. An incision was made into the mucous membrane, and a plane of separation was found between it and the underlying muscle; the mucosa was removed as a sheet by undercutting it with a scalpel in that plane. A

longitudinal strip of the muscle, about 3 cm. long, was taken from the lesser curvature in one experiment, and from the greater in the other.

The test doses of nicotine were left in the bath for 30–60 seconds. The dosage refers not to the base but to the weight of the hydrogen tartrate salt. Hexamethonium was used as the di-bromide or di-iodide ; solutions of these were made from the powder.

RESULTS

Interaction of atropine and nicotine in kittens

Ileum.—Motor responses to 50–100 μg. of nicotine were present in five out of six preparations ; in one of these the response to nicotine was a mixture of contraction and inhibition. The nicotine-contractions were repeatable if the test doses were spaced 4–5 minutes apart. This action of nicotine was in all five experiments reversibly abolished by concentrations of $1\text{–}5 \times 10^{-7}$ of atropine. In favourable circumstances, when tone and rhythmic activity were retained after atropine, an inversion of the nicotine response became manifest (Fig. 1) and could be obtained

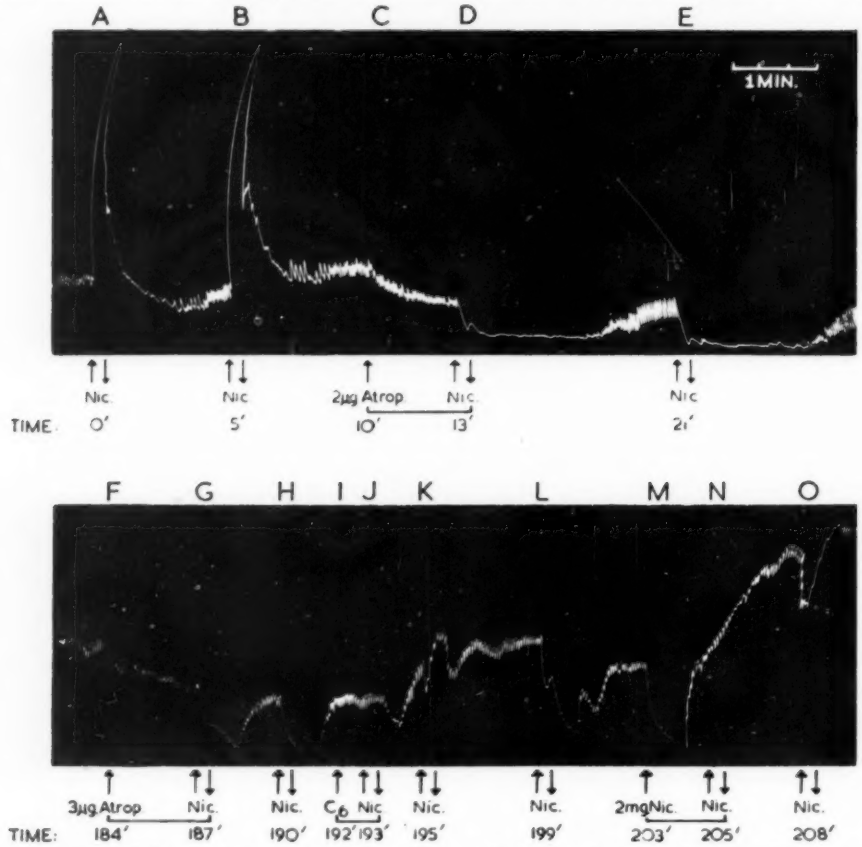


FIG. 1.—Kitten, 0.6 kg.; ileum preparation 40 cm. below pylorus, exhibiting inversion of the nicotine-response in the presence of, and after, atropine. Nicotine (50 μg.) left in the bath for 30 sec. at A, B, D, E, G, H, J, K, L, N, and O; 2 mg. for 120 sec. at M. Atropine (2 μg.) at C, and 3 μg. at F, introduced 3 min. before D and G respectively. Hexamethonium bromide (1 mg.) at I, 60 sec. before J. The minutes below, in this and subsequent figures, refer to the times of administration of each drug. For explanation see text.

repeatedly. Thus in four of the five experiments nicotine produced active inhibition of the gut, both in the presence of atropine and for some considerable time after it had been washed out (Fig. 2) ; eventually the gut recovered from the atropine

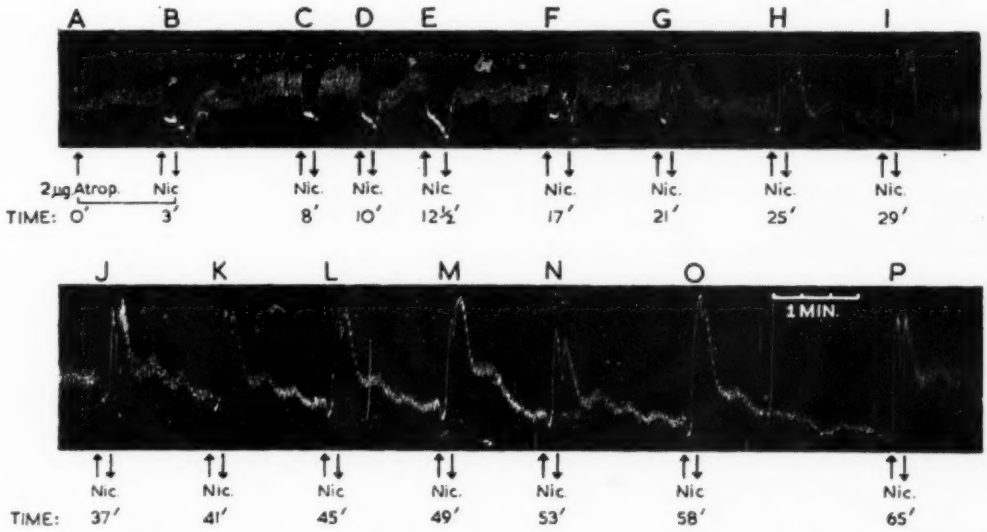


FIG. 2.—Kitten, 0.5 kg.; ileum preparation 20 cm. below pylorus. At A, 2 μg. atropine, left in the bath for 3½ min. At B, 50 μg. nicotine in the presence of atropine. During the next 65 min. at the times indicated, C–P, 50 μg. doses of nicotine, each left in the bath for 30 sec. (except E and F, 45 sec.). Note gradual decrease (after E) in size and duration of the inhibitory component, and growth of the motor component during recovery from atropine.

and the response to nicotine became motor, but could be reversed again with atropine. In the fifth experiment the gut relaxed, and small inhibitory responses to nicotine could be obtained only after the first two doses of atropine ; after the third dose of atropine the motor response to nicotine was abolished as usual, but there was little or no visible sign of inhibition owing to the already relaxed state of the gut.

In the sixth experiment, inhibitory responses to nicotine were recorded from the gut *without* atropinization ; 5–50 μg. nicotine produced a mixture of inhibition and contraction, but 100 μg. produced pure inhibition (Fig. 3).

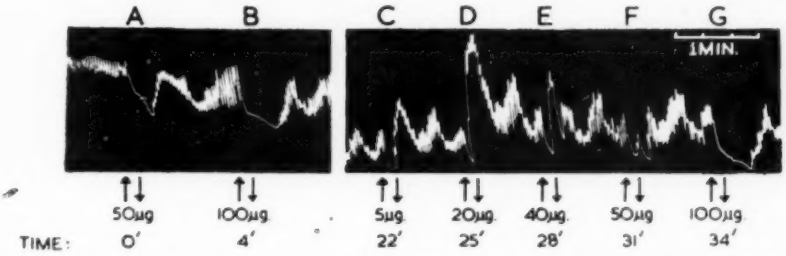


FIG. 3.—Kitten, 0.6 kg.; ileum preparation 29 cm. below pylorus, mounted 85 min. before A, but not subjected to any treatment during that interval. Inhibitory responses to nicotine *without* previous atropinization: the response to nicotine at A–G (each dose given for 30 sec.) is diphasic with 5–40 μg. (C, D, and E) but predominantly inhibitory with 50 μg. (A and F) and with 100 μg. (B and G).

As in the botulinized rabbit gut (Ambache, 1951) the inhibitory response to nicotine of the cat's ileum appears to be due to stimulation of inhibitory ganglion-cells because it is reversibly abolished after ganglionic block with 0.5–1 mg. hexamethonium salts or with 2 mg. nicotine (Fig. 1). As before, the observation was made that the inhibitory response to nicotine is antagonized by 1–2 mg. ephedrine, again suggesting that the inhibitory neurones concerned may be adrenergic.

Stomach strips.—The results of the experiments on the stomach strips from kittens confirm those on the ileum. This muscle is of interest because it was on it that McSwiney and Robson (1929) first demonstrated reversal by atropine of the effect of vagal stimulation in isolated nerve-muscle preparations. We are indebted to Prof. Robson for the information that this experiment, which is illustrated in Fig. 3 of their paper, was conducted entirely on preparations from cats ; the concentration of atropine which they used lay between 10^{-6} and 10^{-5}. Substituting ganglionic

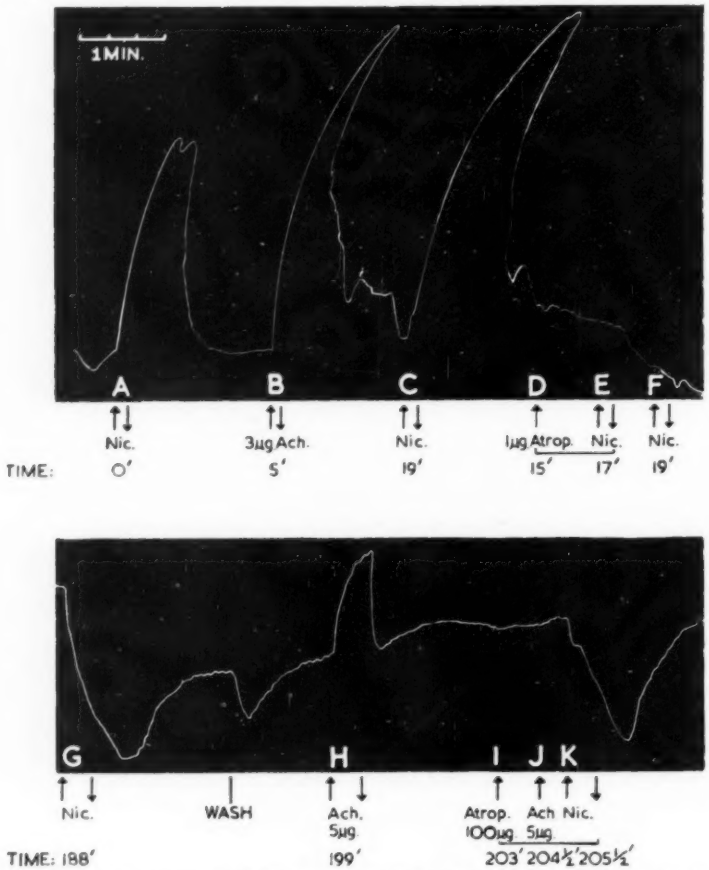


FIG. 4.—Kitten, 0.48 kg.; stomach strip from greater curvature, exhibiting nicotine-reversal after atropine. 200 µg. nicotine at A, C, E, and F, each for 30 sec.; and at G and K, each for 60 sec. Acetylcholine, 3 µg. at B, and 5 µg. at H and J. Atropine, 1 µg. introduced at D, 2 min. before the nicotine at E, and 100 µg. at I left in for 2½ min. till the end of K. The first dose of atropine at D abolished the motor response to nicotine, but nicotine-reversal was not marked until later. In the interval between F and G the muscle was treated several times with atropine, the last dose being 100 µg. atropine for 3½ min., 53 min. before G.

excitation by nicotine for the preganglionic stimulation used by McSwiney and Robson, we have been able to imitate pharmacologically the effect which they observed.

In the present experiments the stomach strips responded by contraction to 100–200 μg. nicotine ; this response was abolished by atropine in concentrations of 10^{-7} in one experiment and 10^{-6} in the other. Nicotine-reversal was not detectable after the first treatment with atropine because of the relaxation of the strips, but after subsequent doses of atropine (1–100 μg.) tone was regained and nicotine-inversion was observed several times (Fig. 4). When present, the inhibitory effect of nicotine could be abolished by 1 mg. hexamethonium.

Non-reversal of the nicotine-response in atropinized rabbit's intestine

It was not possible to obtain a reversal of nicotine-action in rabbit's intestine because the motor response to nicotine persisted in the presence of even large amounts of atropine. As the dose of atropine was raised the response to nicotine diminished in size, but even at an atropine concentration of 10^{-4} remnants of nicotine-contraction were still present (Fig. 5). Pure inhibitory responses to nicotine were

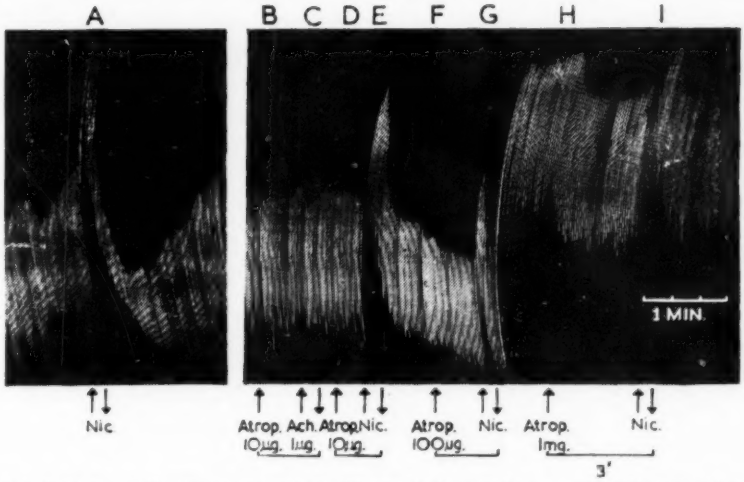


FIG. 5.—Rabbit, 2.15 kg.; ileum preparation, 30 cm. below pylorus, exhibiting persistence of motor response to nicotine in the presence of atropine (up to 10^{-4}). 50 μg. nicotine for 30 sec. at A, E, G, and I. Atropine, 10 μg. at B and D, 100 μg. at F, and 1 mg. at H (3 min. before the nicotine at I). Acetylcholine, 1 μg. at C. Interval of 110 min. between A and B; drum stopped between G and H, with change in baseline.

never recorded, although occasionally the nicotine-contraction was preceded by a loss of one or two beats in the spontaneous rhythm.

This result is based on 20 trials carried out in three different experiments, with atropine-concentrations ranging between 10^{-7} and 10^{-4}. In 15 of the trials the atropine preceded the nicotine by 30 seconds, in the other five by 3–4 minutes. The test dose of nicotine was 50 μg. in eight of the trials, in the other 12 it was reduced to 5 or 10 μg., as the gut was very sensitive. When low doses of nicotine were used, it was sometimes possible to abolish with atropine the contraction produced by 5 μg. nicotine, but reversal did not occur.

Discussion

The reversal of nicotine-action by atropine, like the reversal which is produced by botulinum toxin (Ambache, 1951), may be taken to indicate the presence in the gut of inhibitory postganglionic ("terminal") neurones, which respond to stimulation of their ganglion-cells by inducing relaxation of the gut. For a fuller discussion of the nature of these neurones the reader is referred to the earlier paper (page 60) in which a certain amount of evidence was presented for believing that these intrinsic inhibitor neurones may be adrenergic. It is only necessary to add that a migration of sympathetic neuroblasts from the neural crest to the prevertebral primordium and into the enteric plexus has been described by van Campenhout (1930) in an embryological study on frogs. The same primordium gives rise to the adrenal chromaffin tissue and to the ganglia of the sympathetic chain, i.e., to sympathin-containing nervous tissues. The *extrinsic* inhibitory fibres which the gut receives from the splanchnic nerves are known to be adrenergic; if it should be confirmed that the *intrinsic* inhibitors function by releasing the same transmitter, this identity between the two inhibitory innervations of the gut would provide a good example of economy in design.

In comparing the present results with those obtained previously on botulinized intestines the following agreements and discrepancies become apparent. Reversal of nicotine-action by atropine occurs in preparations from cats but not from rabbits. However, reversal of nicotine-action can occur in rabbits, but only after the motor nerve-endings in the gut have been paralysed with botulinum toxin; atropine (up to 10^{-4}) fails to reverse the action of nicotine because of the persistence, in its presence, of the motor effect of nicotine. Thus it is clear that atropine block of

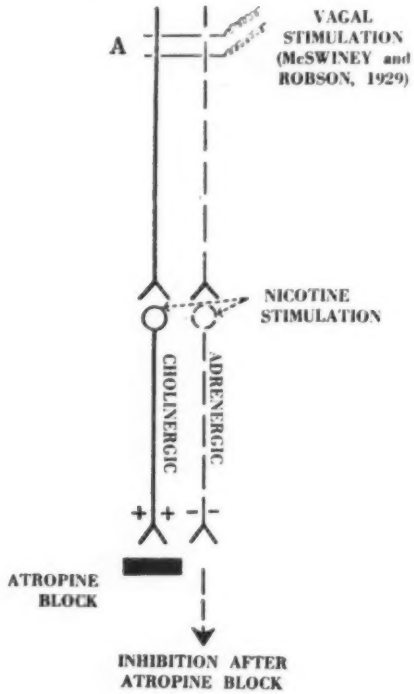


FIG. 6.—Diagram illustrating the possible analogy between vagal-reversal (Bayliss and Starling, 1899; McSwiney and Robson, 1929) and nicotine-reversal, after atropine.

the motor nerve-endings is for some reason effective in cats but not in rabbits; concerning other species little is known except that the nicotine-contraction of the guinea-pig gut is atropine-sensitive (Emmelin and Feldberg, 1947; confirmed by Ambache and Rocha e Silva, 1951, unpublished).

In considering the experiments on the stomach strips a useful parallel may be drawn between the reversal by atropine of the nicotine response, which we observed, and the effect found by McSwiney and Robson (1929) in isolated vagus nerve-stomach preparations. Their stimulus was applied to the preganglionic fibres (Fig. 6 at A), ours to the terminal ganglion-cells themselves. In both series of experiments the response was motor before, and inhibitory after, the atropine-block. If, following a suggestion first made by Langley (1922), we assume that the vagus links up with two distinct sets of postganglionic neurones, one motor (cholinergic) and the other inhibitory, then the analogy between vagal-reversal after atropine and nicotine-reversal becomes evident; this is set forth diagrammatically in Fig. 6.*

It would be of interest to repeat McSwiney and Robson's (1929) experiment on vagus-stomach preparations from rabbits; on the basis of the above analogy vagal-reversal by atropine would be expected to be, like nicotine-reversal, unobtainable in the rabbit because, as we have seen, the latter exhibits atropine-resistance, at least in the intestine. It is beyond the scope of this paper to discuss the nature of this resistance, for which the reader is referred to page 141 of Dale and Gaddum's paper (1930), but the present experiments do show that it is a matter subject to species-variations.

Summary

1. In isolated intestinal preparations, and in stomach strips taken from young kittens, atropine-concentrations of $1–5 \times 10^{-7}$ abolish nicotine-contractions and reveal an inhibitory action of nicotine. This action is ganglionic, since it is abolished by hexamethonium; it is also abolished by ephedrine ($1–2 \times 10^{-4}$).

2. Nicotine-reversal by atropine could not be detected in preparations of rabbit's ileum because the motor response to nicotine persisted in the presence of atropine (10^{-7} to 10^{-4}).

3. An analogy is drawn between (*a*) atropine-induced nicotine- and vagal-reversals and (*b*) the nicotine-reversal produced by botulinum toxin (Ambache, 1951). The three phenomena appear to indicate the presence of inhibitory postganglionic neurones in the wall of the gut.

References

Ambache, N. (1951). *Brit. J. Pharmacol.*, **6**, 51.
Bayliss, W. M., and Starling, E. H. (1899). *J. Physiol.*, **24**, 99.
van Campenhout, E. (1930). *J. exp. Zool.*, **56**, 295.
Dale, H. H., and Gaddum, J. H. (1930). *J. Physiol.*, **70**, 109.
Emmelin, N., and Feldberg, W. (1947). *J. Physiol.*, **106**, 482.
Langley, J. N. (1922). *J. Physiol.*, **56**, 39*P*.
McSwiney, B. A., and Robson, J. M. (1929). *J. Physiol.*, **68**, 124.

* *Note added in proof.*—An alternative arrangement to that shown in Fig. 6 would be that the inhibitory fibres in the vagus are "through-fibres." If that were so, paralysing doses of nicotine, after blocking the peripheral synapse in the motor pathway of the vagus, should produce vagal reversal. Since this does not occur (see, for example, Bayliss and Starling, 1899, p. 143, who state explicitly that the action of the vagus is "permanently abolished" by nicotine) it may be concluded that there exists a peripheral synaptic relay, not only in the motor, but also in the inhibitory pathway of the vagus, as shown in Fig. 6.

Brit. J. Pharmacol. (1951), **6**, 318.

ENZYMES AND THEIR SUBSTRATES IN THE ADRENAL GLAND OF THE OX

BY

H. LANGEMANN*

From the Department of Pharmacology, University of Oxford

(Received March 19, 1951)

Since the formation and the release of pressor agents are the chief functions of the adrenal medulla, it seemed of interest to investigate the activities of enzymes which may be connected with these two functions in the adrenal gland itself.

Ox adrenal glands were used throughout because the medulla and the cortex can be separated fairly easily, the cortex being but little folded and the medulla being very thick and containing few inclusions of cortical tissue. The proportion of cortex to medulla is small, being of the order of about 1.5 to 1.

Since the innervation of the medulla is cholinergic, cholinesterases were determined. The medulla is known to contain a fairly active enzyme hydrolysing acetylcholine, whereas the cortex shows little activity (Antopol and Glick, 1940 ; Langemann, 1942). Amine oxidase activity and histaminase (" diamine oxidase ") activity were also measured, since these enzymes may influence the production and degradation of aromatic pressor amines and their release into the blood stream by histamine. The presence of amine oxidase in sheep adrenal glands has previously been described by Bhagvat, Blaschko, and Richter (1939), and in the medulla and cortex of ox by Schapira (1945). The former authors made no attempt to separate the medulla from cortex, and the latter author used only a qualitative method. Extracts from mammalian kidney (Holtz, Heise, and Lüdtke, 1938) and liver (Blaschko, 1939) are able to form pressor amines like hydroxytyramine (Holtz *et al.*, 1938) and *nor*adrenaline (Blaschko, Burn, and Langemann, 1950), and it was therefore of interest to find out if similar processes occurred in the adrenal gland. Schapira (1946), working with extracts of whole adrenal glands of the guinea-pig, was not able to demonstrate the presence of DOPA-decarboxylase.

METHODS

The easiest and quickest way of separating medulla from cortex was by freezing the gland at $-15°$ C. after its removal from the ox, and separating the darker coloured cortex from the lighter medulla by means of a scalpel. To test the relative completeness of the separation, extracts of the cortex and of the medulla were made and tested on the arterial blood pressure of a spinal cat. One such medullary extract was found to give a blood pressure rise equivalent to 12.5 mg. adrenaline per g. of fresh tissue, whereas the cortical tissue extract had only about 1 per cent of the pressor activity of the medullary extract.

* With a grant of the Swiss " Stiftung für Biologisch-Medizinische Stipendien."

One of the peculiar features of adrenal medullary tissue is the enormous content of adrenaline and *nor*adrenaline, which amounts to about 10 per cent of its dry weight. Figures are given in Table IV also for the relative percentages of the two main pressor agents, adrenaline and *nor*adrenaline, determined by the method of Burn, Hutcheon, and Parker (1950).

Experiments

Cholinesterases.—The tissues were ground in a mortar with sand or silica and then suspended in Krebs's bicarbonate-Ringer. The cholinesterase activity was determined manometrically at 37.5° C. in a gas mixture of 95 per cent N_2 and 5 per cent CO_2, 2.0 ml. of tissue suspension, containing either 200 mg. wet weight medulla or 400 mg. wet weight of cortex, being used. The side bulb contained 0.3 ml. of substrate. The final concentrations used were: acetylcholine bromide 0.026 M, acetyl-β-methylcholine chloride 0.039 M, and benzoylcholine chloride 0.0078 M. The initial reading was taken 3 or 5 min. after tipping, and subsequently at 3 or 5 min. intervals for the following 30 minutes.

The figures for q_{CO_2} given in Table I represent the μl. CO_2 liberated by 1 g. (wet weight) of tissue per 60 min. These values were calculated from 3–15 min. or 5–15 min. after tipping, i.e., when the enzymic hydrolysis was proceeding at a linear rate. The readings were corrected for spontaneous hydrolysis of the substrates and for the extract blanks, which were small.

Addition of 0.0023 M-*l*-adrenaline or 0.0046 M-*dl-nor*adrenaline to a very active preparation of dog's caudate nucleus resulted in an 11 per cent inhibition of the hydrolysis of acetylcholine.

TABLE I

CHOLINESTERASE ACTIVITY OF ADRENAL TISSUE EXTRACTS

Preparation	q_{CO_2} values (μl. CO_2/g. wet tissue/60 min.) for different substrates		
	Acetylcholine	Acetyl-β-methylcholine	Benzoylcholine
Medulla:			
Exp. 1	1,950	590	50
,, 2	2,650	1,470	0
,, 3	2,020	1,050	0
Cortex:			
Exp. 1	230	120	35
,, 2	115	15	0
,, 3	290	90	0

Amine oxidase.—The tissues were ground with sand in a mortar and suspended in M/15 phosphate buffer, *p*H = 7.4. Some preparations were dialysed against phosphate buffer for 3 or 22 hours at 4° C.; this procedure does not affect the amine oxidase activity. The enzymic activity was measured manometrically at 37.5° C. in O_2. Into 1.6 ml. of tissue suspension, containing 400 mg. (wet weight) of tissue, 0.4 ml. of 0.05 M-tyramine hydrochloride or -*iso*amylamine hydrochloride was tipped and readings were taken during the subsequent 30 to 60 min. The CO_2 was absorbed by 0.3 ml. N-NaOH in the centre well of the flask.

The figures given in Table II are expressed as q_{O_2}, i.e., the μl. O_2 consumed by 1 g. (wet weight) of tissue per 60 min., for the blanks and for the experimental flasks. The q_{O_2} was calculated from the initial rate of oxidation, while the reaction was approximately linear. The blanks were often high, especially in cortex, but in this tissue they were reduced almost to zero by dialysis. In medullary suspensions dialysis was less effective in reducing the blanks. This may be due to oxidation of

TABLE II

AMINE OXIDASE ACTIVITY IN OX TISSUE EXTRACTS

Oxygen uptake of tissue suspensions without and in presence of substrate; q_{O_2} = μl.O_2/g. tissue/60 min. In the last two columns blank oxygen consumption has been deducted

Preparation	Exp. No.	q_{O_2} blank	q_{O_2} Tyramine	q_{O_2} *Iso*amylamine
Medulla	1	247	418	—
	2	97	140	—
	3	237	225	50
	4	225	262	141
	5*	160	530	150
	6	260	350	150
	7*	180	590	160
Cortex	1	96	—	64
	2	168	274	—
	3	350	125	(19)
	4	330	250	90
	5*	20	730	70
	6	660	300	200
	6a†	190	380	60
	7*	40	640	100
Kidney	1	125	3,180	1,370
	2*	30	3,180	1,620
Liver	1	180	3,030	1,380
	2*	180	4,140	1,770

* Dialysed extracts (22 hours). † Dialysed extracts (3 hours).

aromatic amines (adrenaline and *nor*adrenaline) which had not been removed completely by dialysis. For comparison, figures for the amine oxidase activity in similarly prepared suspensions from liver and kidney of the same species have been included in Table II; these figures show that the activity of the adrenal tissue is much lower than that of liver or kidney.

Histaminase ("diamine oxidase").—These experiments were carried out in the same way as those for the measurement of amine oxidase, with the difference that cadaverine (pentamethylene diamine) dihydrochloride was used as a possible substrate and that the extract was centrifuged for 5 min. at 2,000 rev. per min. in order to remove particulate matter. The supernatant was used in the manometric experiments.

As in the experiments on amine oxidase, the oxygen uptake of the blanks was high, but there was no excess uptake of oxygen in the presence of cadaverine. Histaminase seems to be absent from these tissues or, at least, only present in amounts too small to be measured by the method employed.

DOPA-decarboxylase.—The method used was that described by Blaschko (1942); the tissue was ground with sand in a mortar, suspended in an equal amount of M/15 phosphate buffer, *p*H = 7.4, and centrifuged. The rate of decarboxylation was followed manometrically at 37.5° C. under nitrogen atmosphere. To 1.6 ml. of the supernatant, corresponding to 800 mg. of fresh tissue, 0.4 ml of a 0.01 M solution of L-DOPA was tipped from the side bulb of a conical manometer flask at the beginning of the experiment. Readings were taken 3 or 5 min. after tipping and then at short intervals, usually up to 30–60 min. until the reaction had come to a standstill. In order to determine the CO_2 retention in the suspension, 0.3 ml. N-HCl from the centre well of the flask was then mixed with the contents. In some experiments semicarbazide was added to give a concentration of 0.01 M, in order to inhibit the enzyme reaction.

The figures for q_{CO_2} given in Table III represent the μl. CO_2 formed by 1 mg. (wet weight) tissue in 60 min. The blanks were usually low. In most of these experiments the q_{CO_2} values were calculated from the first 3 or 5 min. readings after tipping. This was necessary, as the initial rate of the reaction was not maintained for long. Although the initial activity was rather high and comparable to that of guinea-pig kidney, the reaction appeared to come to a standstill before all

TABLE III

DECARBOXYLATION OF DOPA BY OX ADRENAL TISSUE

The figures give the q_{CO_2} per mg. of tissue per 60 min. The values for the blanks, which were small, have been subtracted from these figures

Medulla	The following results were obtained under the experimental conditions described in the text. The q_{CO_2} were: Experiment 1 .. .. .. 0.775 2 .. .. .. 0.800 3 .. .. .. 0.900 4 .. .. .. 0.825 5 .. .. .. 0.825 Mean result from 20 experiments, including some in which amount of tissue, amount of substrate, *p*H, and buffer were slightly modified: q_{CO_2} = 0.695 (lowest figure, 0.226; highest, 1.150)
Cortex	Mean result from 10 experiments: q_{CO_2} = 0.077 (lowest, 0; highest, 0.250)
Kidney (same species) ..	q_{CO_2} = 0.250
Liver (same species) ..	q_{CO_2} = 0.250

the substrate had been decarboxylated. Fifteen experiments were carried out with the cortical extracts and thirty with extracts of medulla. These experiments include many in which the experimental conditions were slightly modified from those described above. Tissue concentration and *p*H were varied, but no higher rates of decarboxylation were obtained. A summary of these experiments is given in Table III.

Since it is known that DOPA-decarboxylase is a pyridoxal phosphate protein, it seemed likely that the enzyme might be inhibited by carbonyl reagents. This

was demonstrated in experiments with extracts from ox liver and kidney. In the presence of 0.01 M-semicarbazide, the DOPA-decarboxylase activity was inhibited by about 90 per cent. The CO_2 formation from DOPA in the presence of an extract of suprarenal medulla was similarly reduced.

Discussion

The experiments on cholinesterase activity of the adrenal gland are of interest, as they show that most of the high activity is localized in the medulla, the cortex containing only about a tenth of that activity. The enzyme is of the type which Mendel, Mundell, and Rudney (1943) described as "true" cholinesterase and Augustinsson and Nachmansohn (1949) as "acetylcholine esterase." This is in agreement with the observation by Ord and Thompson (1950) on the rat suprarenal, but these authors did not separate the medulla and the cortex, whereas the earlier investigators, who separated these tissues, did not at that time differentiate the two types of enzyme (Antopol and Glick, 1940; Langemann, 1942). In the ruminant, "pseudo" or "non-specific" cholinesterase is said to be absent (Gunter, 1946). This is confirmed by our observation on the adrenal gland of the ox, where the hydrolysis of benzoylcholine did not occur or was very small. Acetyl-β-methylcholine, however, was actively hydrolysed. The experiments reveal an interesting difference between the cholinesterase of adrenal tissues in the ox and those of the whale (Burn, Langemann, and Parker, 1951): in the latter species acetylcholine is hydrolysed rapidly, but acetyl-β-methylcholine only very slowly and benzoylcholine not at all.

The activity of amine oxidase in both medulla and cortex was found to be rather low in comparison with that in liver or kidney of the same species. Moreover, the small amount of tissue of the adrenal glands, compared with that of other organs with high amine oxidase activity, would mean that the adrenals contribute little to the total turnover rate for amines in the animal body. The experiments with dialysed extracts seem to indicate that the adrenal gland has a markedly higher activity towards tyramine as compared with *iso*amylamine. This may be considered as an adaptation of the enzyme to the type of amines usually present in the gland.

Since histamine is known to release pressor amines from the adrenal gland (Burn and Dale, 1926), the histaminase (diamine oxidase) activity was measured, but no activity of extracts of adrenal medulla or of cortex was found.

It may be of interest to put together (Table IV) the concentrations of the substrates of some of the enzymes here described which have been found to be present in medulla and cortex of the ox adrenal. It may be mentioned that choline oxidase activity was looked for, but not found in our experiments.

DOPA-decarboxylase activity in the adrenal medulla is particularly interesting. The activity is as high as that of the guinea-pig kidney, known to be the best source for this enzyme. The enzymic activity is about ten times higher in adrenal medulla than in cortex. It is also several times higher in the medulla than in the kidney or in the liver of the ox. This suggests strongly that DOPA-decarboxylase has an important function in the medullary tissue and that, in spite of the relatively small size of the adrenal gland, the enzymic activity in this tissue represents a significant portion of the total activity of this enzyme in the body of the ox.

Holtz *et al.* (1938) showed that the decarboxylation of DOPA by extracts of mammalian kidney resulted in the production of a pressor amine, hydroxytyramine.

TABLE IV

CONCENTRATIONS OF ENZYME SUBSTRATES IN OX ADRENAL TISSUE

Figures are given as μg. per g. wet tissue

Substrate	Gland No.	Medulla	Cortex
Adrenaline and *nor*adrenaline	1	12,500 (75%)*	140
	2	11,400 (50%)*	—
	3	16,500 (50%)*	—
Histamine (5 experiments) estimated by the method of Code (1937)		1.1–9.1 2.6 (mean value)	0.15–1.35 0.6 (mean value)
Acetylcholine†		0.6–0.72	0.12–0.2
Choline†		50	170

* The percentage means % adrenaline present together with *nor*adrenaline measured by the method of Burn, Hutcheon, and Parker (1950).

† These figures are taken from Feldberg and Schild (1934).

Hydroxytyramine has only about 1/35th of the pressor activity of adrenaline, which makes it difficult to demonstrate the formation of this amine in the presence of the large amounts of adrenaline and *nor*adrenaline found in these extracts.

The fact that the enzymic decarboxylation of DOPA by medullary tissue extracts did not reach completion in any experiment, as it usually does with kidney extracts, may be due to inhibition by the reaction product. Blaschko (1942) found that the addition of the reaction product, hydroxytyramine, in a molar concentration ten times that of the substrate DOPA inhibited the reaction by 71 per cent. These findings were extended by Polonovski, Schapira, and Gonnard (1946), who reported that equimolar amounts of *nor*adrenaline, as well as sympatol (synephrin) and tyramine, inhibited the DOPA-decarboxylase activity of guinea-pig kidney extracts by 61–65 per cent. The possibility cannot therefore be excluded that the adrenaline or *nor*adrenaline present in medullary extracts in very high concentration caused an inhibition of the DOPA-decarboxylase. It seems worth considering whether this inhibition may not act as a regulating mechanism in the living animal; after physiological stimulation, when the content of pressor amines in the gland is relatively low, this enzyme might be more active than at rest, when the gland is full of these substances.

It has been shown that DOPA can be rapidly decarboxylated by the adrenal medulla (and less actively by the cortex), giving rise, very probably, to hydroxytyramine. It is known that with guinea-pig kidney extracts dihydroxyphenylserine can also be decarboxylated, although less rapidly than DOPA, and that *nor*adrenaline is formed in this reaction (Blaschko, Burn, and Langemann, 1950). It seems likely that in the kidney one and the same enzyme is responsible for the decarboxylation of the two amino-acids. These experiments therefore raise the question whether *nor*adrenaline can similarly be formed by decarboxylation in the adrenal medulla of the ox.

The DOPA-decarboxylase determinations were made in a nitrogen atmosphere. This is necessary, as in an oxygen atmosphere the amine oxidase present in the

same tissue extract would further degrade amines formed by decarboxylation. The experiments by Bing (1941) showed that kidneys, perfused with DOPA under hypoxic conditions, form a pressor substance which is similar in its action to hydroxytyramine. Amine oxidase, which is present in kidney in high activity, requires a good oxygen supply, and therefore was unable to destroy the amines formed in these experiments. In experiments on the perfused adrenal gland of the dog, Bülbring, Burn, and Elío (1948) showed that oxygen lack resulted in an output of pressor amines (adrenaline and *nor*adrenaline were not determined separately) into the venous blood. Under these conditions, therefore, the activity of the amine oxidase present in the gland can be expected to be low.

The experiments on the enzyme activities found in the adrenal gland suggest the possibility that the formation and the release of the pressor amines depend on the interaction of amine oxidase and DOPA-decarboxylase in the adrenal medulla itself, regulated in their functional state by the oxygen supply of the blood.

Summary

1. Extracts of ox adrenal medulla and cortex have been prepared and examined for their enzymic activities.

2. The cholinesterase activity of the medulla was high, that of the cortex about one-tenth of the medullary extracts ; the enzyme hydrolysed not only acetylcholine but also acetyl-β-methylcholine ; the hydrolysis of benzoylcholine was negligible.

3. The amine oxidase activities of the medullary and cortical extracts were of about the same order ; tyramine was oxidized more rapidly than *iso*amylamine. There was no evidence of the occurrence of histaminase (diamine oxidase).

4. L-DOPA was found to be decarboxylated at a high rate in extracts of medulla.

I wish to thank Professor J. H. Burn for the hospitality of his laboratory and Dr. H. Blaschko for his advice and interest during this work.

References

Antopol, W., and Glick, D. (1940). *J. biol. Chem.*, **132**, 669.
Augustinsson, K. B., and Nachmansohn, D. (1949). *Science*, **110**, 98.
Bhagvat, K., Blaschko, H., and Richter, D. (1939). *Biochem. J.*, **33**, 1338.
Bing, R. J. (1941). *Amer. J. Physiol.*, **132**, 497.
Blaschko, H. (1939). *J. Physiol.*, **96**, 50P.
Blaschko, H. (1942). *J. Physiol.*, **101**, 337.
Blaschko, H., Burn, J. H., and Langemann, H. (1950). *Brit. J. Pharmacol.*, **5**, 431.
Bülbring, E., Burn, J. H., and de Elío, F. J. (1948). *J. Physiol.*, **107**, 222.
Burn, J. H., and Dale, H. H. (1926). *J. Physiol.*, **61**, 185.
Burn, J. H., Hutcheon, D. E., and Parker, R. H. O. (1950). *Brit. J. Pharmacol.*, **5**, 142.
Burn, J. H., Langemann, H., and Parker, R. H. O. (1951). *J. Physiol.*, **113**, 123.
Code, C. F. (1937). *J. Physiol.*, **89**, 257.
Feldberg, W., and Schild, H. (1934). *J. Physiol.*, **81**, 37P.
Gunter, J. M. (1946). *Nature, Lond.*, **157**, 369.
Holtz, P., Heise, R., and Lüdtke, K. (1938). *Arch. exp. Path. Pharmak.*, **191**, 87.
Langemann, H. (1942). *Helv. Chim. Acta*, **25**, 464.
Mendel, B., Mundell, D. B., and Rudney, H. (1943). *Biochem. J.*, **37**, 473.
Ord, M. J., and Thompson, R. H. S. (1950). *Biochem. J.*, **46**, 346.
Polonovski, M., Schapira, J., and Gonnard, P. (1946). *Bull. Soc. chim. biol., Paris*, **28**, 735.
Schapira, J. (1945). *C.R. Soc. Biol., Paris*, **139**, 36.
Schapira, J. (1946). *C.R. Soc. Biol., Paris*, **140**, 173.

Brit. J. Pharmacol. (1951), **6**, 325.

THE MODE OF ACTION OF ANTRYCIDE

BY

W. E. ORMEROD

From the National Institute for Medical Research, Mill Hill, London, N.W.7

(Received March 21, 1951)

This paper deals with the mode of action of "Antrycide"* on trypanosomes *in vitro* and in the living animal. Although the particular virtue of antrycide is its activity against *T. congolense*, in the experiments reported here a strain of *T. equiperdum* which has been maintained in this laboratory for routine drug standardization was used. This strain has the advantages that it produces a constant acute infection with high blood parasite counts in rats and mice, that it is fatal for untreated animals within a period that can be predicted according to the degree of infection, and that it survives *in vitro*. Although Curd and Davey (1950) showed that there are large quantitative differences in the susceptibility of species of trypanosomes to antrycide there is no reason to suppose that any qualitative differences exist except between the susceptible strains and *T. cruzi* which they found insusceptible to antrycide. In this paper, when the species of trypanosome is not mentioned *T. equiperdum* was used. Where it was necessary to make sure that any particular finding was not an individual strain characteristic, strains of *T. rhodesiense* and *T. congolense* were used.

I should like to thank Messrs. Imperial Chemical (Pharmaceuticals), Ltd., for a gift of antrycide methylsulphate; this salt has been used throughout, except where otherwise stated.

How the resistant strain of *T. equiperdum* used in these experiments was formed will be described in a later publication.

EXPERIMENTS

The effect of antrycide on trypanosomes in vitro

Trypanosomes were incubated at 37° C. with varying concentrations of the drug. The method used, based on that of Yorke, Adams, and Murgatroyd (1929), was as follows:

A rat showing trypanosomes in the blood in the order of 20 per high-power microscopic field was killed by immersion in pure CO_2. Blood taken from the heart was suspended in sterile 1.5 per cent citrate saline, to prevent clotting, and a washing fluid consisting of 1 volume horse serum to 9 volumes Ringer's solution. This mixture was centrifuged lightly in order to remove blood cells and leave the trypanosomes in the supernatant fluid, which was then put into fresh vessels. The trypanosomes were centrifuged down and resuspended in the final diluting fluid, which consisted of 4 volumes Ringer's solution to 1 volume pooled serum from normal rats. A drop of this fluid containing

* Trade mark of Imperial Chemical (Pharmaceuticals) Ltd.

trypanosomes was then added to each of a series of tubes containing varying concentrations of antrycide in 0.2 per cent glucose Ringer's solution. The trypanosomes were counted at intervals, and their infectivity estimated by injecting the whole contents of a tube into a mouse.

A typical experiment is shown in Table I. Very little trypanocidal action can be seen in experiments such as that recorded in Table I, where the trypanosomes were taken from a rat with rising infection. There was, however, a more marked but somewhat irregular loss of infectivity which could also be demonstrated when the

TABLE I

ACTION OF ANTRYCIDE ON TRYPANOSOMES *in vitro*

Table showing a typical experiment with a normal strain of *T. equiperdum* incubated with antrycide. The figures in the column headed "Trypanosomes × 1,000 per cu.mm." represent the concentration in each tube, which contained 0.25 c.c. of fluid; this was injected into a mouse at the time of counting

++ Mouse infected within three days. + Mouse infected within 10 days. — Mouse uninfected

Concentration of antrycide	Results after incubation for							
	5 min.		2½ hr.		4½ hr.		20 hr.	
	Trypanosomes × 1,000 per cu.mm.	Infectivity	Trypanosomes × 1,000 per cu.mm.	Infectivity	Trypanosomes × 1,000 per cu.mm.	Infectivity	Trypanosomes × 1,000 per cu.mm.	Infectivity
1/20,000 ..	36	++	22.0	—	19.2	—	0	—
1/40,000 ..	36	—	24.8	—	22.0	—	3.6	—
1/80,000 ..	36	++	34.8	+	26.4	+	2.0	—
1/160,000 ..	36	++	10.8	+	35.2	—	9.2	—
1/320,000 ..	36	++	36.0	+	26.0	+	9.2	—
1/640,000 ..	36	++	35.6	—	23.6	+	2.8	—
Control ..	36	++	36.4	++	30.4	++	4.4	+
Control ..	36	++	30.8	++	31.6	++	4.0	+

trypanosomes were washed free from antrycide-containing fluid. In other experiments where the infection was further advanced, high concentrations of the order of 1/80,000 killed a proportion of the trypanosomes; this effect varied widely from one experiment to another both in the number of trypanosomes killed and in the time taken for the effect to appear. Trypanocidal activity was never observed at concentrations as low as those achieved *in vivo*, i.e., of the order of 1/1,000,000 (Spinks, 1950).

Because of the variability of results with normal strains of trypanosomes it is not possible to give any strict comparison of results with resistant strains. There was, however, no detectable difference between normal and resistant strains either in their susceptibility to trypanocidal action or in their loss of infectivity.

The serum of a rat 24 hours after an effective dose of antrycide showed no activity against trypanosomes *in vitro*.

The effect of antrycide on the respiration of trypanosomes *in vitro* was measured by the Warburg technique. Whole blood from the heart of a rat killed by CO_2 was treated with sufficient citrate saline to prevent clotting; this was added to the main compartment of the Warburg flask, which already contained the required dilution of

antrycide in Ringer's solution with 0.2 per cent glucose. The final number of trypanosomes was 1–2 thousand million per 3 c.c. fluid in the main compartment of each flask ; the centre compartment of each flask contained 0.2 c.c. 20 per cent (w/v) KOH.

No inhibition was observed with concentrations of antrycide less than 1/80,000, and the normal decline in oxygen consumption when the glucose in the medium was used up occurred at the same time as in controls. Some inhibition was, however, observed at 1/40,000 and varied in different experiments from 20–50 per cent of controls at one hour from the beginning of the experiment. Inhibition was also observed at the same concentration with a resistant strain.

It is unlikely that either the inhibition of respiration or the trypanocidal effect *in vitro* is in any way connected with the therapeutic effect of antrycide ; it is more likely to be an incidental physical effect such as the precipitation of antrycide chloride on the cell membrane. The limit of solubility of antrycide chloride in Ringer's solution at 37° C. is approximately 1/25,000, and it is readily precipitated from saline solutions made from the methylsulphate.

Action of antrycide in vivo

Infected mice were dosed intraperitoneally with antrycide in distilled water ; the trypanosomes in the blood continued, however, to increase after dosing, although after an effective dose the rate of increase was less than in controls. A large dose given in an early infection could prevent the rise in trypanosome count, although this was often difficult to achieve in mice owing to the toxicity of the drug. After a normal curative dose a peak of infection was reached after 24–48 hours and the blood cleared of trypanosomes in 2–4 days. Table II shows the response of an

TABLE II

RESPONSE OF AN INFECTION WITH *T. equiperdum* TO ANTRYCIDE

The antrycide was given intraperitoneally on the first day of infection (LD50 intraperitoneal antrycide for Parkes strain mice $\simeq$ 0.34 mg./20 g.)
R = Relapsed on the 9th day. N = Negative for 15 days. + Died

Days of Infection	Extent of infection after varying doses of antrycide in terms of trypanosomes per field or per 20 fields							
	0.05 mg./20 g.		0.1 mg./20 g.		0.2 mg./20 g.		Controls	
1	7/1	3/1	2/20	2/1	16/20	9/1	6/1	6/20
2	16/1	7/1	2/1	7/1	1/1	8/1	20+/1	14/1
3	10/1	8/1	2/1	5/1	3/20	5/1	+	+
5	0/20	0/20	0/20	0/20	0/20	0/20		
>5	R	N	N	N	N	N		

infection of 0.05 mg./20 g., a dose after which some relapses occur ; to 0.1 mg./20 g., a normal curative dose ; and to 0.2 mg./20 g., the maximum dose that can be given without mortality.

Trypanosomes may remain in the blood for several days after a dose of antrycide, but their infectivity is markedly reduced, as is shown in Table III. In Exp. A an ineffective dose was given to the donor, and the result was only a slight loss of infectivity ; in (B) after an effective dose the infectivity of the trypanosomes was diminished 3/4 and 4 hours after dosing, and was abolished after 24 hours, so that none of the mice injected at that time became infected in 17 days.

TABLE III

EFFECT OF ANTRYCIDE ON INFECTIVITY

Table showing the proportion of mice that became infected after passage from donor animals dosed with antrycide. Donor animals were infected (20 trypanosomes per field) and passage was effected at various intervals after dosage with antrycide. (A) represents an ineffective and (B) an effective dose. Within each group a standard number of trypanosomes was injected into each mouse

Donor species and dose	Days after passage	Controls	Mice infected/mice injected at intervals after antrycide				
			½ hr.	¾ hr.	3 hr.	4 hr.	24 hr.
A Mouse 0.04 mg./20 g.	1 2 4 5	3/3	9/9		7/8 8/8		0/10 0/10 5/10 9/9 (one mouse died)
B Rat 0.1 mg./20 g.	2 4 10 17	10/10		0/10 0/10 3/10 4/10		0/10 0/10 1/10 6/10	0/10 0/10 0/10 0/10

Morphological changes in trypanosomes treated with antrycide

Although trypanosomes treated with antrycide appear normal when examined in wet films, their loss of infectivity indicates that they must have undergone some change. Serial counts were therefore made, using stained dry smears, in order to detect any possible change in the rate of cell division. The trypanosomes were counted as: (1) Normal resting forms. (2) Dividing forms; for the purposes of the count, "dividing forms" included all trypanosomes showing divided blepharoplasts, or two flagellae, or a nucleus showing a pale band across the middle caused probably by the migration of chromatin to its opposite poles. (3) Binuclear forms showing two nuclei.

Table IV shows one such series of counts. The variability from animal to animal and from experiment to experiment is considerable, particularly in regard to the number of binuclear forms in trypanosomes from treated animals. However, the three following features, besides the gradual death of the trypanosomes, were unmistakable in this and two similar experiments:

1. The appearance of mis-shapen cells damaged presumably in making the slide.

2. A general decrease in the number of dividing forms; this was not apparent until late in the experiment when the trypanosome counts were beginning to fall.

3. The appearance of deep violet staining inclusion bodies in the cytoplasm in groups or large masses, often cramming the cell and obliterating other features of the internal structure. These were the first abnormalities to appear in treated trypanosomes, and were present after 24 hours in every animal that had received a therapeutic dose: They persisted as long as trypanosomes remained in the blood, but occasionally at the end of the third or on the fourth day the highly degenerate trypanosomes remaining in the blood did not contain inclusion bodies but showed

TABLE IV

Table showing: 1. Degree of infection in trypanosomes per field or per 20 fields. 2. The percentage—resting, dividing, binuclear, and mis-shapen forms in three mice treated with 0.05 mg./20 g. antrycide and three control mice. 3. The appearance of inclusion bodies noted with a star. 0.05 mg. antrycide per 20 g. produced inclusion bodies in all the trypanosomes and cleared all trypanosomes from the blood in 120 hr.

	Forms	At 47 hours 0.05 mg /20 g. antrycide was given							
		Hours after infection							
		44		68		72		92	
		Degree of infection	% Forms	Degree of infection	% Forms	Degree of infection	% Forms	Degree of infection	% Forms
Treated mice	Resting	2/1	80	5/1	71	4/1	79	1/1	62
	Dividing ..		15		20		9		0
	Binuclear ..		5	*	9	*	7	*	12
	Mis-shapen ..		0		0		12		24
	Resting	7/20	90	3/1	86	4/1	71	15/20	66
	Dividing ..		15		1		1		2
	Binuclear ..		5	*	7	*	4	*	0
	Mis-shapen ..		0		6		24		32
	Resting	1/1	82	15/20	75	3/1	80	2/1	78
	Dividing ..		11		13		4		4
	Binuclear ..		7	*	9	*	1	*	11
	Mis-shapen ..		0		3		15		7
Control mice	Resting ..	9/20	80	20+/1	89	20+/1	91	20+/1	67
	Dividing ..		13		10		8		22
	Binuclear ..		7		1		1		11
	Mis-shapen ..		0		0		0		0
	Resting ...	3/1	85	14/1	82	20+/1	75	20+/1	63
	Dividing ..		15		16		17		32
	Binuclear ..		0		2		7		5
	Mis-shapen ..		0		0		0		0
	Resting	12/20	68	15/1	85	20+/1	75	20+/1	66
	Dividing ..		29		9		17		32
	Binuclear ..		3		6		8		2
	Mis-shapen ..		0		0		0		0

very pale cytoplasm, as though, in losing the inclusion bodies, staining material had gone out of the cell. In occasional mice, maximal doses of antrycide failed to produce inclusion bodies and the cells degenerated without undergoing any specific change. An ordinary curative dose always produced inclusion bodies in the normal strains of *T. equiperdum*, *T. congolense*, and *T. rhodesiense* used. After such a dose all trypanosomes were to some degree affected, but their appearance differed widely in animals of the same species treated similarly; they were produced equally well in rats and mice. After an ineffective dose only a fraction of the trypanosomes contained inclusion bodies; this appearance was also produced in a partially resistant strain. With a totally resistant strain no inclusion bodies were produced.

These inclusion bodies, shown in Plates 1 and 2, stained a deep violet with the usual eosin-methylene blue compound stains, freshly prepared Leishman giving the best results; the cytoplasm retained its usual basophilia but in some instances appeared paler than usual. They were not usually visible in unstained trypanosomes except with phase contrast microscopy, when in the living and actively motile trypanosome they appeared as highly refractile granules contrasting with the paler

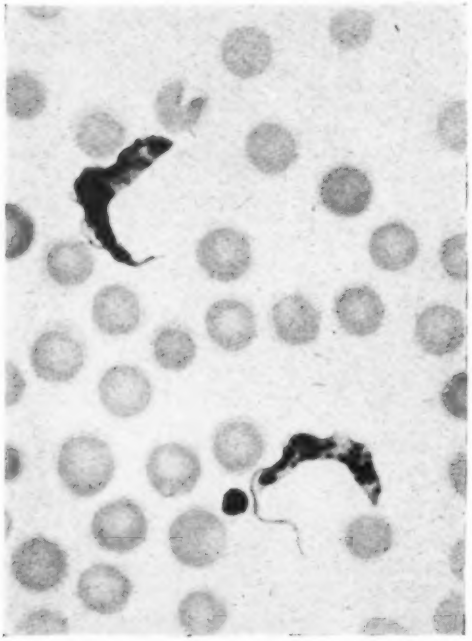


PLATE 1.—*T. equiperdum* in rat. Inclusion bodies produced by antrycide, 10 mg./kg. Stained with Leishman.

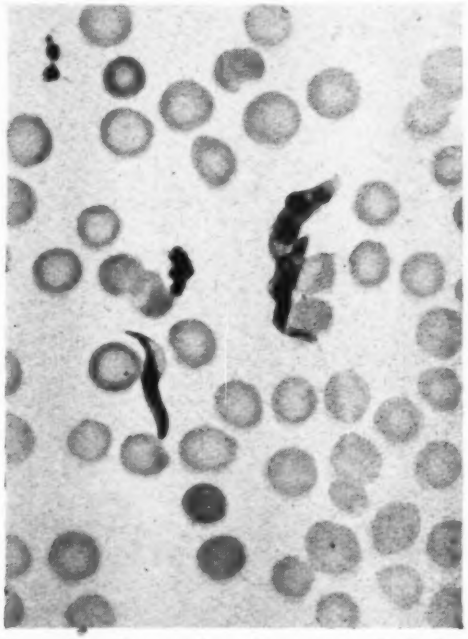


PLATE 2.—*T. congolense* in mouse. Inclusion bodies produced by antrycide, 1.0 mg./kg. Stained with Leishman.

cytoplasm and nucleus. They did not seem in any way to impede the trypanosomes, which were normal except for these inclusion bodies and a slight distension of the pale basal vacuole. A similar appearance can be seen in the dried unfixed preparation under the fluorescence microscope, when trypanosomes from an animal treated with antrycide show faintly fluorescent bodies (see Plate 3). The largest in some trypanosomes corresponds to the basal vacuole, but the others have a similar distribution to the inclusion bodies as shown by other methods, and it is most probable that the remainder of the absorbed antrycide is concentrated in these inclusion bodies. No fluorescence could be detected in the nucleus of the trypanosome.

Multinuclear forms

In the experiment shown in Table IV and in other similar experiments with *T. equiperdum*, cells with more than two nuclei were only seen on three occasions. *T. rhodesiense* appears, however, to produce multinuclear forms more readily. In the strain used, 0.05 mg. antrycide per 20 g. was necessary to clear the blood of

PLATE 3.—*T. equiperdum* in mouse. Fluorescent inclusion bodies produced by antrycide, 2.5 mg./kg. Fluorescence photomicrograph taken with an Osira lamp, Woods glass filter transmission maximum 3650A, and sensitive emulsion exposed for one hour. The fluorescent drug can be seen concentrated in the inclusion bodies.

PLATE 4.—*T. rhodesiense* in mouse. Stained with Leishman. Multinuclear forms produced by antrycide, 0.5 mg./kilo. A few of these abnormal cells are produced in the blood of a mouse treated with 1/5th dose necessary to clear the blood. Division of the cell wall is inhibited, but internal structures continue to divide.

trypanosomes, but in mice that had been given one-fifth of this dose, up to 5 per cent of the trypanosomes had more than two nuclei. The trypanosome in Plate 4 is an extreme example with six nuclei, three blepharoplasts, and four flagellae, showing that division of the cytoplasm and cell membrane has been inhibited by the drug, but the internal structures have continued to divide.

DISCUSSION

As the molecule of antrycide is large and contains two permanent positive charges, it seemed likely that its action was on the surface of the trypanosome in the manner of a cationic detergent. On the surface membrane antrycide might act either by inhibiting surface enzymes, or indirectly by covering the trypanosome and "starving it out." The first of these possibilities is easily eliminated by the delay before any activity can be observed in animals given a dose of antrycide; an indirect action on the surface is less easily eliminated, but it is most likely that trypanosomes affected in this way would be moribund, whereas when inclusion bodies are fully developed in trypanosomes, and their infectivity abolished by a dose of antrycide, they are still actively motile and cannot be distinguished from normal trypanosomes in the ordinary wet film. Finally, trypanosomes that have been treated with antrycide show a faint fluorescence which comes chiefly from structures similar to those that appear in the cytoplasm in stained preparations.

All this strongly suggests that antrycide penetrates slowly into the cell and exerts its action there and not upon the surface membrane.

There is no indication that the primary action of antrycide is upon the nucleus. The evidence suggests either that nuclear changes occur as a result of cytoplasmic changes or that antrycide penetrates the nuclear membrane after affecting the cytoplasm: (*a*) Inhibition of nuclear division occurs late in the cycle of events. (*b*) Cell division can be inhibited independently of nuclear division. (*c*) Fluorescence of antrycide has not been observed in the part of the cell occupied by the nucleus. There is, however, strong evidence that the drug acts upon the cytoplasm where abnormal inclusion bodies can be seen in the living trypanosome under phase contrast at a time when similar, if not identical structures, show the fluorescence of antrycide in the dry unfixed film.

There is no evidence to suggest that antrycide inhibits enzymes or the utilization of metabolites (Town, Wills, and Wormall, 1949), although no experiments *in vitro* have been successfully carried out on trypanosomes in the late stages of contact with the drug when it will have been absorbed. But whether or not it acts by inhibiting cell metabolism it is clear that it is a growth inhibitor rather than a direct trypanocide ; this is shown by: (*a*) Absence of trypanocidal effect *in vitro*. (*b*) In *T. rhodesiense* a small dose of antrycide can inhibit cell division while nuclear division continues. (*c*) Trypanosomes can remain circulating in the blood and actively motile after a dose of antrycide that has produced structural changes and rendered them non-infective ; all vital functions appear intact except the ability to reproduce.

Summary

1. Antrycide has been shown to be without trypanocidal action *in vitro* at higher concentrations than can be achieved *in vivo* during observation periods up to 20 hours.

2. Similar high concentrations have no observable effect *in vitro* on the respiration and glucose utilization in trypanosomes.

3. Still higher concentrations of the order of 1/20,000 which do show some trypanocidal activity *in vitro* and inhibit the respiration of trypanosomes are equally active against normal and antrycide-resistant trypanosomes.

4. An effective dose of antrycide may take from two to four days to clear trypanosomes from the blood of a mouse, but after 24 hours the circulating trypanosomes will not infect fresh mice.

5. After an effective dose, basophilic inclusion bodies appear in the cytoplasm of all trypanosomes in the blood 24 hours after dosing. These bodies can also be seen in the living trypanosome by phase contrast microscopy.

6. Twenty-four hours after a dose of antrycide, faintly fluorescent bodies can be seen in the cytoplasm of trypanosomes. It is suggested that some of the largest of these correspond to the distended basal vacuole and the others to the inclusion bodies mentioned above.

7. From 24 hours after an effective dose the number of trypanosomes showing signs of division diminishes slowly.

8. *T. rhodesiense* reacts to a small dose of antrycide by producing multinuclear forms.

9. Three conclusions are put forward and discussed:

(*a*) Antrycide penetrates slowly into the cell and exerts its action in the cell and not on the surface membrane.

(*b*) The action of antrycide is on the cytoplasm and not primarily on the nucleus.

(*c*) Antrycide in normal curative doses acts as a growth inhibitor and not as a direct "trypanocide."

I should like to express my thanks to Dr. F. Hawking, who proposed that I should investigate the mode of action of antrycide, for his interest and stimulating criticism; to Mr. J. Smiles and his staff for their interest and co-operation, to which I owe the illustrations and much of the substance of this work; and to my assistants Mr. D. Cragoe, Mr. F. Jabir, and Mr. C. Hill.

References

Curd, F. H. S., and Davey, D. G. (1950). *Brit. J. Pharmacol.*, **5**, 25.
Spinks, A. (1950). *Brit. J. Pharmacol.*, **5**, 445.
Town, B. W., Wills, E. D., and Wormall, A. (1949). *Nature, Lond.*, **164**, 233.
Yorke, W., Adams, A. R. D., and Murgatroyd, F. (1929). *Ann. trop. Med. Parasit.*, **23**, 501.

Brit. J. Pharmacol. (1951), **6**, 334.

A STUDY OF BASOPHILIC INCLUSION BODIES PRODUCED BY CHEMOTHERAPEUTIC AGENTS IN TRYPANOSOMES

BY

W. E. ORMEROD

From the National Institute for Medical Research, Mill Hill, London, N.W.7

(Received March 21, 1951)

In the previous paper a description was given of inclusion bodies produced in trypanosomes by antrycide. In this paper a more detailed study of the chemical behaviour of these inclusion bodies and a discussion of their significance will be given in three parts.

I. Methods by which inclusion bodies can be produced in trypanosomes and other types of cell, and their relation to basophilic granules that occur naturally in the cytoplasm of trypanosomes.

II. A study of the nature of these inclusion bodies by cytochemical techniques.

III. A mode of action for certain " trypanocidal " drugs.

I. Methods by which inclusion bodies can be produced

(a) By trypanocidal drugs

Of the drugs that have so far been examined *in vivo*, antrycide, dimidium bromide, and suramin produced well-marked inclusion bodies, tryparsamide and stilbamidine did not produce basophilic inclusion bodies, acriflavine (a mixture of proflavine and its N-methyl derivative) caused the trypanosomes to degenerate so that although inclusion bodies could be detected they were not well formed and may have been altogether different structures.

Only two references to inclusion bodies produced in trypanosomes by chemotherapeutic agents have been found in the literature. Saito (1928) described granules in trypanosomes after treatment (presumably *in vivo*) with acriflavine, and these probably correspond to the ill-defined basophilic inclusion bodies produced by this drug which were referred to above. Lavier (1928) described " Paravacuolar formations " which were produced " in certain crises (unspecified) resulting in a cure for the host animal," and he distinguished them by vital staining from the naturally occurring " Volutin," " Chromatoid," or " Metachromatic " granules. Lavier's work is difficult to evaluate both for lack of experimental details and because the vital stains that he used to distinguish these two types of formation—pyronin, thionin, and methylene blue—can themselves create artifacts with properties similar to those that he was investigating, as will be shown later in this paper.

(b) By vital stains and other non-trypanocidal basic substances

An experiment designed to investigate the staining properties of these inclusion bodies in the living trypanosome was performed as follows: Trypanosomes showing inclusion bodies from an animal treated with antrycide were examined under the microscope in the wet smear with oil immersion objective, and a saturated solution of dye was added under the coverslip. Various dyes were used; those containing primary, secondary, or tertiary amine groups stained the inclusion bodies, and, of these, neutral red and toluidine blue were the most effective. No acid or quaternary amine dye was found which stained living trypanosomes. Under these conditions the inclusion bodies were the only structures in the trypanosome which were stained.

Normal trypanosomes were also stained by this technique and produced a most striking reaction. The dye was at first distributed evenly in the cytoplasm; then after about a minute (depending on the final concentration of dye in the diluting medium) the coloured cytoplasm gradually formed stained aggregates which were identical in appearance and distribution with the stained inclusion bodies produced in trypanosomes from an antrycide treated animal. The production of inclusion bodies in this way by neutral red (a concentration of 1/1,000,000 dye in Yorke's medium produced them in one hour) was found to be reversible, that is to say, if the trypanosomes containing these coloured granules were centrifuged free of neutral red containing solution, and resuspended in fresh diluting fluid, their appearance became normal when viewed both by ordinary and by phase contrast microscopy. When 40 mg. neutral red per 20 g. was given by intravenous injection to a mouse heavily infected with trypanosomes, and blood taken five minutes later and examined in the wet unstained smear, inclusion bodies could be seen with the same appearance and distribution as those produced by neutral red *in vitro*. After 30 minutes only a few of these inclusion bodies could be seen, and after 60 minutes they had all disappeared. In dry fixed smears inclusion bodies produced in this way could be stained with eosin-methylene blue compound stains; their staining properties were similar to those of the inclusion bodies produced by antrycide, except that the violet colour which they assumed was slightly less intense.

The formation of basophilic granules by basic substances in cells other than trypanosomes *in vitro* and *in vivo* is a phenomenon that has been studied intensively by other workers and has been reviewed by P. Dustin (1947). The studies have been made chiefly on nucleated red cells and reticulocytes but are applicable to many other cell types, the only requisite for the production of basophilic granules being a basophilic cytoplasm. Dustin identified this basophilic substance in nucleated red cells as ribonucleo-protein and the chief component of the basophilic granules as ribonucleic acid.

(c) Naturally occurring inclusion bodies or "Volutin" granules

L. van den Berghe (1942) investigated "Volutin" granules in a strain of *T. gambiense* which produced large numbers with great regularity and identified their chief component as ribonucleic acid, but as his only method of identification involved the use of non-crystalline ribonuclease his conclusion must be accepted with reserve. I have not been able to verify his findings for lack of a strain of trypanosomes that produces "Volutin" granules with regularity, but can find no

evidence to suggest that in their chemical nature the "Volutin" granules differ from inclusion bodies produced by drugs or by vital stains. It is probable that the "Volutin" granules (which occur only with certain strains of trypanosomes and in certain species of host) are an unspecific reaction of the trypanosome to non-trypanocidal basic substances circulating in the blood.

II. A CYTOCHEMICAL STUDY OF INCLUSION BODIES PRODUCED BY ANTRYCIDE

Mice infected with *T. equiperdum* showing 1 to 10 trypanosomes per oil immersion field were injected with antrycide methylsulphate (0.02 mg./20 g.), and smears were taken on glass and quartz slides when inclusion bodies had appeared in the cytoplasm of the trypanosomes. At 24 hours the inclusion bodies were fully developed and the trypanosomes otherwise normal. At 48 hours the inclusion bodies were the same, but the trypanosomes as a whole showed signs of degeneration.

The smears were fixed unless otherwise stated by heating at 160° C. for 20 seconds and by immersion when cool for two minutes in methanol.

Staining with Unna's carbol-pyronin-methyl green.—Slides (fixed by immersion for two minutes in methanol) were placed for twenty minutes in this solution at 37° C. The trypanosome nuclei stained a faint green and the inclusion bodies a full orange red.

Kurnick (1950) has shown that the polymerized state of desoxyribonucleic acid causes it to stain green specifically with methyl green. The orange red staining of ribonucleic acid with pyronin is not so specific, but in the presence of an intact nucleus staining with methyl green it indicates that the inclusion bodies are composed of cytoplasmic material probably containing ribonucleic acid.

Staining with Giemsa after treatment with specific enzymes.—As a general indication of the chemical composition of cells, a basophilic reaction to eosin-methylene blue compound stains shows the presence of nucleic acid, and an eosinophilic reaction shows the presence of protein (Pollister, 1950). More specific information can be gained by enzyme digestion of smears and subsequent staining with Giemsa, which is in this instance the most satisfactory variety of this general type of stain.

Incubation was performed at 30° C. in twice distilled water containing the enzyme, and a control preparation was treated in each experiment in the same way but with the enzyme omitted. This technique was employed because buffered solutions rapidly removed basophilic material from the cytoplasm, but pure water removed basophilia less rapidly; a strict comparison with the control was always necessary to determine how much of the decrease in basophilia could be attributed to the enzyme.

Crystalline ribonuclease (Kunitz, 1940) in 0.04 per cent solution completely removed the inclusion bodies and cytoplasmic basophilia in 15 minutes. This preparation is probably not protease-free but may contain traces of pancreatic proteolytic enzymes.

Protease-free crystalline ribonuclease (Macdonald, 1948) at similar concentration removed cytoplasmic basophilia, but failed in 60 minutes to remove the inclusion bodies. It did, however, alter their appearance, and they stained more eosinophilic and less basophilic than controls.

Crystalline trypsin in 0.04 per cent solution had no effect after 60 minutes' treatment on the staining of trypanosomes, but when combined with an equal amount of the above solution of protease-free ribonuclease the inclusion bodies were removed in 15 minutes. The appearance of the nucleus in all these experiments was unchanged.

Although these results are clear-cut it must be emphasized that the interpretation of individual experiments is a matter largely of judgment owing to the unpredictable behaviour of these enzymes and the fact that controls also showed diminished staining through the action of the distilled water. Yet, in conjunction with the confirmatory tests given below, it can safely be taken that the chief constituents of the inclusion bodies are ribonucleic acid and protein.

Confirmatory tests for the presence of ribonucleic acid and protein in the inclusion bodies

Gram stain.—Henry and Stacey (1943) have shown that the Gram staining material of bacteria is magnesium ribonucleate. The specificity of this test for ribonucleates is doubtful since the reaction mechanism is unknown. The reagents used were crystal violet, and iodine in potassium iodide solution, and the decolorization with absolute alcohol gave the best contrast after 20 seconds. The inclusion bodies were Gram positive.

The Feulgen reaction to distinguish desoxyribonucleic acid from ribonucleic acid was not strictly applicable since the acid digestion necessary for the Feulgen reaction was sufficient to remove all basophilic material from the cytoplasm and the inclusion bodies. The nuclei, however, remained on the slide and gave a faintly positive Feulgen reaction for desoxyribonucleic acid.

Coupling with tetrazotized benzidine (Mitchell, 1942 ; Danielli, 1947).—Of the substances likely to be present in the cell, tyrosine, histidine, and derivatives of pyrimidine are susceptible to attack by a diazonium reagent. The reactivity of tyrosine and histidine can be blocked by benzoylation, and the pyrimidine derivatives, which are not benzoylated, will be stained a reddish brown by the following technique:

> Smears are left for 12 hours at room temperature in a solution of 10 per cent (w/v) benzoyl chloride in pyridine, washed well and left for half an hour in 0.05 per cent tetrazotized benzidine in barbiturate buffer at *p*H 9, washed with three changes of buffer and left for 45 minutes in saturated sodium bicarbonate saturated with β-naphthol. The coupling reactions are performed at 0–4° C.

The dye is formed in the nucleus and inclusion bodies of the trypanosomes, indicating the presence of pyrimidine combined with the cell structure ; as vitamins, coenzymes, or other trace substances containing pyrimidine derivatives will have been washed away by this treatment, this is a sensitive cytochemical test for both types of nucleic acid. The pyrimidinium nucleus of antrycide is not attacked by diazonium reagents.

Ultra-violet absorption.—The inclusion bodies absorb heavily in photographs taken at wavelength 2750A. This indicates a concentration of purines and pyrimidines in the inclusion bodies ; this would be a valuable test for nucleic acid but for the pyrimidinium nucleus of antrycide which also absorbs at this wavelength.

Millon's tyrosine reaction.—Unfixed smears were left for 12 hours in Bensley's modification of Millon's reagent (Bensley and Gersh, 1933). The inclusion bodies appeared as dark refractile granules; the outline of the trypanosome could not be seen under the microscope by visual light, but was apparent in photographs taken with a fluorite objective, mercury vapour lamp, and Wood's glass filter with transmission in the 3650A region, which is the absorption maximum of the Millon tyrosine complex (Pollister, 1950).

III. A MODE OF ACTION FOR TRYPANOCIDAL DRUGS WHICH PRODUCE BASOPHILIC INCLUSION BODIES

In the previous paper evidence was put forward that antrycide inhibited the growth of trypanosomes and that it entered the trypanosome and was concentrated in the inclusion bodies. Although previous workers have not drawn the same conclusion for dimidium bromide (Lock, 1950) and suramin (Hawking, 1939; Janscó and Janscó, 1934) there is much evidence that their mode of action is similar. This may be summarized as follows.

1. Chemotherapeutic concentrations are without trypanocidal activity *in vitro*.

2. Normal effective doses may take several days to clear the blood of an infected animal.

3. Chemical estimation of the suspension fluid has either failed to detect absorption of the drug by trypanosomes or detected only minute amounts.

4. Inclusion bodies are produced by all three types of drug after 24 hours at normal effective dose levels.

Strong circumstantial evidence therefore exists that these three drugs are growth inhibitors rather than direct trypanocides and that they enter the trypanosome in small amounts and are concentrated in the inclusion bodies.

It is, of course, by no means certain that the production of inclusion bodies by these drugs is not independent of their trypanocidal activity, and that some other as yet undetected process is not of more fundamental importance, but I believe that the most cogent reason against there being an inhibition of any vital process caused by these drugs is the unimpaired motility of trypanosomes that have absorbed an effective dose. A more satisfactory explanation of their activity is provided by the theory—held generally for some time but best expressed in the work of Caspersson (1950)—that the interaction of protein and ribonucleic acid in the cytoplasm is an essential point of protein synthesis, cell growth, and reproduction; so in the drug-treated trypanosome, growth and reproduction cease when an appreciable amount of the protein or ribonucleic acid is inactivated, as it would be by being removed by the drug from the phase of the cytoplasm to that of the inclusion body.

Two important questions, however, remain.

(i) How can suramin, a molecule with six *negative* charges, act in the same way as dimidium bromide and antrycide which have respectively one and two *positive* charges?

(ii) Why should not all substances that can produce inclusion bodies be trypanocidal?

(i) Some of the less complex nucleoproteins can be considered as salt-like complexes of basic protein and nucleic acid (Greenstein, 1944) and like other salts can *in vitro* be broken down by double decomposition with *either* acids *or* bases. It is most probable that this also can occur in the cytoplasm of trypanosomes. As the molecules of these drugs are large, and the molecules of protein and ribonucleic acid still larger, the resulting complex would be precipitated from the phase of the cytoplasm giving the conditions necessary for a double decomposition of nucleoprotein. Suramin has been shown to form complexes with the basic groups of protein (Wills and Wormall, 1950), dimidium bromide with nucleic acids (Brownlee *et al.*, 1950), and antrycide with acids of high molecular weight (Ormerod, unpublished).

(ii) The charged form of most basic vital stains is in equilibrium at *p*H 7.5 with an uncharged form which enters the trypanosome rapidly. It can also diffuse out again when the outside concentration of the substance is reduced and thereby reverse the reaction between the dye and nucleoprotein. Antrycide, suramin, and dimidium bromide have, however, permanent charges. Antrycide is known to penetrate the cell very slowly, and, as suramin and dimidium bromide produce inclusion bodies at approximately the same rate, they are probably also slow in penetrating the cell. When the external concentration of the drug falls the drug diffuses out of the cell so slowly that the trypanosome may end its normal life span before the processes of growth and division can be resumed. The fundamental difference between the actions of these two classes of substances is that the inclusion bodies produced by vital stains are reversible, and those produced by the trypanocidal drugs treated here are irreversible.

If the cell membrane were thermodynamically " ideal " the rate of diffusion into and out of the cell would be the same, but as a reaction has been demonstrated inside the cell that can fix acidic or basic substances that penetrate the cell membrane, the most probable departure from ideality will favour concentration of these substances in the cell.

Although no inclusion bodies which can be demonstrated in the dry fixed smear have been found in trypanosomes treated with stilbamidine, it is most probable that some similar reaction does in fact occur. Trypanosomes treated with stilbamidine show highly fluorescent granules (Hawking and Smiles, 1941) which resemble the fluorescent granules that appear in the cytoplasm of trypanosomes treated with antrycide. But when stilbamidine treated trypanosomes are stained there are no basophilic inclusion bodies demonstrable. However, in some of the trypanosomes, unstained spaces can be seen, and these give the suggestion that inclusion bodies are present but do not stain with eosin-methylene blue.

Kopac (1945) has postulated a mode of action similar to the one put forward in this paper for stilbamidine against tumour cells ; and inclusion bodies of a similar chemical and histological nature have been demonstrated (Snapper *et al.*, 1947) in myeloma cells from patients treated with stilbamidine, but it is impossible at this moment to fit stilbamidine into the same reaction scheme as antrycide, dimidium bromide, and suramin, for although the hypothesis that I have outlined may describe a mechanism common to the interaction of other drugs and biological systems it is not yet possible to detect it without the appearance of histological changes ; also, when inclusion bodies have appeared it is not possible to prove that they are not subsidiary phenomena unrelated to the main action of the drug. Although it was

demonstrated in the previous paper that antrycide was actually concentrated in the inclusion bodies it may be argued that this is due to the operation of a defence mechanism in the trypanosome removing a noxious substance from the vital parts of the cell ; but whether this is so or not, the concentration of protein, ribonucleic acids, and the drug in the inclusion bodies strongly suggests that the drug has upset the normal combination of protein and nucleic acids, which, if one accepts the theories of Caspersson (1950), may be considered sufficient to inhibit the growth of the trypanosome.

Conclusion

The general conclusion derived from this work must be treated as a working hypothesis, since the theoretical background used to interpret experimental results described in this paper cannot be said to have advanced far beyond a state of hypothesis.

Antrycide, dimidium bromide, and suramin inhibit the growth of trypanosomes by splitting the cytoplasmic nucleoprotein into its constituent protein and ribonucleic acids. It is probable that not all the nucleoprotein in the cytoplasm is so affected. Work on other systems (Greenstein, 1944) suggests that only the simpler forms of desoxyribonucleoprotein are held together by purely electrostatic bonds which can be split by acids or bases without denaturation of protein, and the same will probably apply to the ribonucleoprotein involved in the reaction with antrycide. The reaction with neutral red is completely reversible so that in this instance there can be no protein denaturation ; if the two reactions are fundamentally the same, as I believe them to be, the conclusion is justified that it is only the simpler nucleoproteins present in the trypanosome which are involved. The more complicated nucleoproteins which might not be broken down by this reaction would account for the basophilia that remains in the cytoplasm after the formation of inclusion bodies either by antrycide or neutral red.

The possibility—advanced by Kopac (1945) to explain the mode of action of stilbamidine on tumour cells—that the trypanocidal drugs treated in this paper act by denaturation of the protein fraction of the cytoplasmic nucleoprotein of trypanosomes, in contrast to the action of non-trypanocidal vital stains which leave the protein undenatured, must be considered. But although this may be a factor in the action of other types of drugs, for reasons which have been stated above, I believe that the action of antrycide, dimidium bromide, and suramin is determined by their possessing electric charges which, once the drugs have penetrated the cell membrane, tend to retain them in the trypanosome, preventing its growth and division.

In this paper the problem of structural specificity of drugs for trypanosomes has not been discussed ; this is, however, one of the ways in which it is hoped that further investigation of inclusion body formations may be fruitful.

Summary

1. Basophilic inclusion bodies are produced in trypanosomes in the living animal by effective doses of antrycide, dimidium bromide, and suramin.

2. Similar inclusion bodies are produced *in vitro* by vital stains, and their formation can be observed under the microscope.

3. The relation of these inclusion bodies to the "Volutin" granules, and to basophilic inclusion bodies produced in other biological systems, is discussed.

4. The inclusion bodies produced by antrycide have been shown to contain ribonucleic acid and protein.

5. It is suggested that these drugs inhibit the growth and division of trypanosomes by preventing the normal interaction in the cytoplasm of protein with ribonucleic acid.

6. The activity of antrycide, dimidium bromide, and suramin is connected with the presence in their molecules of permanent charges so that they are retained in the trypanosome; the charged forms of vital stains, on the other hand, are in equilibrium at *p*H 7.5 with uncharged forms which can diffuse rapidly through the trypanosome membrane.

7. The changes produced by the trypanocidal drugs treated here are permanent, whereas those produced by vital stains are reversible.

In this work I have received valuable help and criticism from many colleagues at Mill Hill. In particular I should like to thank Dr. F. Hawking, Mr. J. Smiles, and Dr. R. Porter, of the National Institute for Medical Research, Dr. J. Craigie, of the Imperial Cancer Research Fund, and my assistant Mr. C. Hill.

References

Bensley, R. R., and Gersh, I. (1933). *Anat. Record*, **57**, 228.
van den Berghe, L. (1942). *Acta biol. belg.*, **4**, 464.
Brownlee, G., Goss, M. D., Goodwin, L. G., Woodbine, M., and Walls, L. P. (1950). *Brit. J. Pharmacol.*, **5**, 261.
Caspersson, T. (1950). *Cell Growth and Cell Function*, New York : Norton.
Danielli, J. F. (1947). *S.E.B. Symposium I*, 101.
Dustin, P., jun. (1947). *S.E.B. Symposium I*, 114.
Greenstein, J. P. (1944). *Recent Advances in Protein Chemistry*, **1**, 209, New York : Academic Press.
Hawking, F. (1939). *Ann. trop. Med. Parasit.*, **33**, 13.
Hawking, F., and Smiles, J. (1941). *Ann. trop. Med. Parasit.*, **35**, 45.
Henry, H., and Stacey, M. (1943). *Nature, Lond.*, **151**, 671.
von Janscó, N., and von Janscó, H. (1934). *Zbl. Bakt.*, (I Abt.) **132**, 257.
Kopac, M. J. (1945). *New York Acad. Sci., Section of Biology*, 5.
Kunitz, M. (1940). *J. gen. Physiol.*, **24**, 15.
Kurnick, N. B. (1950). *J. gen. Physiol.*, **33**, 243.
Lavier, G. (1928). *C.R. Acad. Sci., Paris*, **186**, 401.
Lock, J. A. (1950). *Brit. J. Pharmacol.*, **5**, 398.
Macdonald, M. R. (1948). *J. gen. Physiol.*, **32**, 39.
Mitchell, J. S. (1942). *Brit. J. exp. Path.*, **23**, 296.
Pollister, A. W. (1950). *Rev. Haematol.*, **5**, 527.
Saito, M. (1928). *Trop. Dis. Bull.*, **25**, 801. (Original in Japanese.)
Snapper, I., Mirsky, A. E., Ris, H., Schneid, B., and Rosenthal, M. D. (1947). *Blood*, **2**, 311.
Wills, E. D., and Wormall, A. (1950). *Biochem. J.*, **47**, 158.

Brit. J. Pharmacol. (1951), **6**, 342.

THE PRESSOR AMINES IN THE ADRENAL MEDULLA AFTER IRRADIATION

BY

C. T. ANDERSON, H. BLASCHKO, J. H. BURN, AND R. H. MOLE

From the Department of Pharmacology, Oxford University, and the Medical Research Council's Radiobiological Research Unit, Atomic Energy Research Establishment

(Received April 9, 1951)

There is evidence that the administration of pyridoxine hydrochloride successfully prevents the "radiation sickness" which may appear a few hours after a therapeutic dose of irradiation (Manfield, McIlwain, and Robertson, 1943; Shorvon, 1949). Pyridoxine has also been reported to reduce the mortality of mice given whole body *x*-irradiation (Goldfeder, Cohen, Miller, and Singer, 1948). In seeking for an explanation of this action we considered the relation of pyridoxine to the formation of the pressor amines, *nor*adrenaline and adrenaline, which occur in the adrenal medulla. One of us (Blaschko, 1939) has put forward the view that the enzyme dopa-decarboxylase (dopa is dihydroxy-phenylalanine) discovered by Holtz, Heise, and Lüdtke (1938) plays a part in the formation of *nor*adrenaline. Blaschko, Carter, O'Brien, and Sloane Stanley (1948) have demonstrated that the enzyme is absent in the livers of rats fed on a diet deficient in pyridoxine, and Blaschko, Carter, and Sloane Stanley (unpublished) have shown that the enzyme can be reconstituted in extracts of such deficient livers by adding pyridoxal-5-phosphate *in vitro*.

If it is true that dopa-decarboxylase is an important enzyme in the process of *nor*adrenaline formation, then rats fed on a pyridoxine-deficient diet should have difficulty in maintaining a normal content of pressor amines in the adrenal medulla. Blaschko, Burn, and Carter (unpublished) have found that, when the adrenal medulla is depleted as a result of the injection of insulin, rats fed on a pyridoxine-deficient diet cannot replenish the store of pressor amines so effectively as controls given a supplement of pyridoxine. The inability to replenish the store is, however, only evident in those rats in which the amount of dopa-decarboxylase in the liver is too small to be detected by manometric observations.

The experiments to be described have therefore been directed to examining changes in the amount of dopa-decarboxylase in the livers of irradiated rats, and also changes in the amount of pressor amines in the adrenal medulla both before and after the injection of insulin.

METHODS

Irradiation of rats.—Albino rats from an inbred laboratory stock were irradiated singly or in pairs in an aluminium box. Treatment factors were 240 kv. 15 mamp. H.V.L. 1.14 mm. Cu., tube distance 70 cm., dose rate 42 r./min. The field was uniform to $\pm 3\%$. The rats weighed from 250 to 350 g. and were usually litter mates.

Estimation of dopa-decarboxylase.—The livers were at once frozen in an ice-cooled mortar at almost $-10°$ C. and ground up with a little sand; 0.067 M-sodium phosphate

buffer solution of *p*H 7.4 was then added, 1 ml. for each g. of liver. The resultant suspension was then centrifuged for 5 minutes to remove the sand and connective tissue, and the supernatant was used in the manometric experiments. These were set up as described by Sloane Stanley (1949); in some of our experiments, however, the supernatant was diluted with an equal volume of water in order to reduce the rate of the enzymic reaction and thus to ensure linearity for a longer period.

Estimation of pressor amines.—In the adrenal gland of the normal rat, Burn, Hutcheon, and Parker (1950) found that the amount of pressor amines was 0.92 mg. per g., this being the mean figure for 78 animals, and that 90 per cent was adrenaline, the remaining 10 per cent being *nor*adrenaline. We have followed their procedure in estimating the pressor amines in the irradiated rats. Each rat was killed by a blow on the head; its throat was cut, and it was bled out; the adrenals were dissected quickly from fat and connective tissue and were weighed. They were ground in a mortar with a few grains of sand and 1.0 ml. of 0.02 N-HCl for each 10 mg. gland. This extract was transferred to a centrifuge tube, which was then immersed for 1.5 minutes in a boiling water bath. The tube was cooled, centrifuged, and the supernatant fluid transferred to a sample tube which was fitted with a stopper and kept frozen at −15° C. until the estimation was carried out. The estimation was performed either on the blood pressure of the spinal cat or on an isolated loop of the duodenum of the rabbit (Burn, Finney, and Goodwin, 1950).

RESULTS

Rats killed after three days.—The irradiation given produced signs of its effect after 65–70 hours. The rats were first examined when these signs, disinclination of the rats to move, staring of the fur, were clearly seen. Usually two irradiated rats and two control rats were investigated in each experiment, and the content of pressor amines in the four extracts was determined side by side on the blood pressure of the same cat or on the same loop of rabbit intestine. The pressor amines were estimated in comparison with adrenaline and the amine content was expressed as adrenaline. Table I gives an example of the results of an experiment,

TABLE I

Exp. 4. Rats received 1,000 r. Killed 75 hr. after irradiation

Rat	Body wt. at death (g.)	Adrenal wt. (mg.)	Pressor amine μg. per 100 g. rat	Dopa-decarboxylase μl.CO_2/hr./mg. liver
Control	345	41.9	18.2	0.85
,, ...	275	39.0	18.4	0.86
Irradiated	260	58.5	8.3	0.26
,, ...	250	61.8	9.1	0.62

in which the rats were killed 75 hours after irradiation. Three points are evident; first, that the weight of the adrenal glands of the irradiated rats was nearly 50 per cent greater than that of the control rats; second, that the pressor amine in the adrenals, calculated, not per unit weight of the adrenals, but per 100 g. rat, was about one-half in the irradiated rats of what it was in the controls; third, that the mean figure for dopa-decarboxylase in the irradiated rats was also about half of what it was in the controls.

The other experiments in which these changes were studied are set out in Table II; all the irradiated rats received 1,000 r. and were killed at the times

TABLE II

Exp.	Control rats		Irradiated rats		
	Pressor amine μg./100 g. rat	Dopa-decarboxylase μl.CO_2/hr./mg. liver	For hrs.	Pressor amine μg./100 g. rat	Dopa-decarboxylase μl.CO_2/hr./mg.
1	12.1 20.8		72 72	6.4 16.2	
2	15.3 13.4		73 73	14.5 9.8	
3	17.4 14.1	0.85 0.82	80 80	17.4 4.4	0.45 0.51
5	18 0 18 5	0.51 0.78	78 78	5.8 4.4	0.45 0.48
6	18.7 16 5	0.86 0.28	70 71 71.5	5.0 7.0 5.7	0.48 0.70 0.28
7	14.5 14.0	0 76 0 88	67.5 67.5	10.3 9.1	0.44 0 52
8	14.6	1.35	65.5 65.5	12 8 16.1	1.14 0.95
	18.4	0.65	71.6 71.6	7.8 14.2	0.49 0.50
9	— —	0.55 0.53	72 72	— —	0.48 0 33
Mean	16.4	0.75		9.7	0.53

stated, which varied from 65.5 to 80 hours. In each experiment the mean content of pressor amines in the adrenal medulla was less in the irradiated rats than in the control rats. In one experiment, not included in Tables I and II, when two recently dead rats and one moribund rat were examined, the pressor amine content was less than 5 per cent of the controls. These rats were irradiated 79 hours previously. The mean control figure for all experiments in Tables I and II was 16.4 μg. per 100 g. rat, and the mean figure for irradiated rats was 9.7, the standard errors of these means being ± 0.61 and ± 0.99 respectively. The difference between the means was therefore highly significant. The figures for dopa-decarboxylase in the liver were in every experiment higher in the control rats than in the irradiated rats except in one pair in Experiment 6 where they were the same. The mean figure for control rats was 0.75 and for irradiated rats 0.53, the respective standard errors being ± 0.076 and ± 0.054. The difference between the means was, therefore, highly significant.

Reactivation with pyridoxal-5-phosphate.—It was of interest to find whether the relatively low dopa-decarboxylase activity was due to a reduction in the amount of enzyme protein or of the prosthetic group. The dopa-decarboxylase activity of liver extract from an irradiated rat and its normal control was therefore determined

in the presence and absence of 10 μg. calcium pyridoxal-5-phosphate (synthetic codecarboxylase). This amount of pyridoxal phosphate, which we owe to the kindness of Dr. K. Folkers, restores the enzymic activity of moderately pyridoxal-deficient rats to a normal level (Blaschko, Carter, and Sloane Stanley, unpublished).

The result of one satisfactory experiment of this kind is set out in Table III, which shows that the activity of the extract from the control animal was slightly

TABLE III

Reactivation by added codecarboxylase; 10 μg. calcium-5-pyridoxal phosphate was added to each manometer flask

	Dopa-decarboxylase μl.CO_2/hr./mg. liver	
	Control	Irradiated
Without codecarboxylase	0.73	0.41
With codecarboxylase	0.80	0.78

raised by the addition of synthetic codecarboxylase. In the extract from the irradiated animal the activity in the absence of added codecarboxylase was low, but the addition of dopa decarboxylase raised the activity to a normal figure. We conclude that the low activity in the irradiated animal was not due to low content of enzyme protein, but to a lowering of the codecarboxylase content of the preparation.

Effect of adding pyridoxine to the diet.—The results at this point suggested that the effect of irradiation in causing a fall in the amount of pressor amines in the adrenal medulla might indeed be explained as a consequence of a fall in the amount of dopa-decarboxylase. However, although the irradiated rats had less decarboxylase, the mean fall was to about 70 per cent of the figure for the control rats, that is to say, it was not a fall to zero. The unpublished work of Blaschko, Burn, and Carter, already mentioned, showed that in rats deficient in the decarboxylase as a result of excluding pyridoxine from the diet, the power to restore the pressor amine content of the adrenals after depletion by insulin was significantly reduced only in those rats in which the deficiency was so extreme that no detectable decarboxylase remained.

If the fall in pressor amines after irradiation was due to the lack of dopa-decarboxylase, the administration of pyridoxine should prevent the fall. Experiments were therefore carried out in which pyridoxine was added to the diet two days before irradiation and for the three days after it. Each rat received 1 mg. pyridoxine per day. It was found that this addition of pyridoxine raised the figures for liver decarboxylase to 80 per cent of the figures for the control animals, but that the fall in the pressor amines in the adrenal medulla occurred just as before. The results are shown in Table IV. It was thus clear that the fall in pressor amines in the adrenal glands was not a consequence of the fall in the amount of dopa-decarboxylase in the liver recorded in Table II. The fall in the amount of this enzyme is perhaps related to a diminished absorption of pyridoxine from the intestine, and if this is so the effect of the diminished absorption is evident in as short a time as three days.

TABLE IV

EFFECT OF ADDED PYRIDOXINE

Each rat received 1 mg. pyridoxine daily for 5 days

Exp.	Rat	Body wt. (g.)	Adrenal wt. (mg.)	Time since irradiation	Pressor amine μg./100 g. rat	Dopa decarboxylase μl.CO_2/hr./mg. liver
9	Control	270	48	—	23.3	1.05
	,,	245	47	—	17.4	1.01
	Irradiated	230	60	72 hr.	5.9	0.80
	,,	195	58	75.5	5.7	0.78
10	Control	280	55	—	13.4	0.65
	Irradiated	350	77	58 hr.	10.3	0.56
	,,	250	52	72	8.3	0.53

Pressor amines after insulin.—We have finally made experiments to determine the time at which the adrenal medulla first fails to maintain its store of pressor amines. There is no change to be observed at 48 hours after irradiation as shown in Table V, but a change of another kind can be observed at this time if the rats are injected with insulin. The hypoglycaemia which results causes a liberation of pressor amines from the adrenal medulla, the liberation depending on the degree of hypoglycaemia.

TABLE V

Exp.	Rat	Body wt. (g.)	Adrenal wt. (mg.)	Time since irradiation	Pressor amine μg./100 g. rat
11	Control	295	38	—	14.1
	,, ...	290	41	—	14.1
	Irradiated	270	35	48	13.5
	,, ...	285	43	48	16.6

A recent study of this effect of insulin has been made by Burn, Hutcheon, and Parker (1950), who found that, when rats were kept without food overnight and were then given 0.2 unit insulin per 100 g. body weight by subcutaneous injection, the total amount of pressor amine in the adrenal medulla fell to 60 per cent of the original amount in four hours after the injection. Rats vary greatly in the degree of hypoglycaemia caused by a given amount of insulin, the variation depending on the time of year, on their diet, and other factors. We found that 48 hours after irradiation the injection of a given amount of insulin produced fewer symptoms in the irradiated rats than in control rats, and that in consequence the adrenals of the irradiated rats were less depleted of their content of pressor amines than those of the controls when examined four hours after the injection. An example is given in Table VI. In this experiment two control rats and two irradiated rats were kept without food overnight, and insulin in a dose of 0.2 unit per 100 g. rat was injected 44 hours after irradiation into all four rats. Two hours later a second injection of half this amount was given to all the rats, which after a further two hours were then killed. The control rats were much more affected by the insulin than the irradiated rats. One control rat had convulsions, and the other was collapsed. Neither irradiated rat showed symptoms. The amounts of pressor amine in the

TABLE VI

RATS INJECTED WITH INSULIN. KILLED 4 HR. LATER

Exp.	Rat	Body wt. (g.)	Adrenal wt. (mg.)	Time since irradiation	Pressor amine μg./100 g. rat
12	Control	270	37	—	4.1
	,, ...	290	43	—	5.9
	Irradiated	280	37	48	15.8
	,, ...	300	43	48	11.5

adrenal medulla were in accordance ; thus the amounts were very low in the control rats, and were almost normal in the irradiated rats. Thus at 48 hours the irradiated rats were much more resistant to insulin than the control rats, although at this point there was no adrenal hypertrophy.

The results in a series of such experiments carried out at varying times after irradiation are given in Table VII. In each experiment one or two control rats were

TABLE VII

THE ADRENAL GLANDS OF IRRADIATED RATS INJECTED WITH INSULIN

Note that the figures for pressor amine in the third column indicate in Exps. 12–16 greater depletion in the controls, and in Exps. 17–18 greater depletion in irradiated rats

Exp.	Time after irradiation (hr.)	Pressor amine as % of control	Adrenal wt. as % of control
12	48	273	100
13	48	116	90
14	56	169	110
15	60	134	108
16	64	242	112
17	68	37	164
18	72	42	132

compared with two irradiated rats, and the irradiated rats were killed at the time after irradiation stated in the second column. The mean amount of pressor amine in the irradiated rats is stated as a percentage of that in the control rats, both groups having been injected with a given dose of insulin per 100 g. four hours previously. (If the control rats showed no symptoms two hours after the injection, a further injection of insulin was then given to all rats.) In the last column the weights of the adrenals of the irradiated rats are expressed as a percentage of the weights of those of the control rats. The method of drawing up Table VII can be seen from the figures for Experiment 12, which are given in detail in Table VI and in summary in Table VII. The latter Table shows that, up to 64 hours after irradiation, no deficiency in pressor amines in the adrenal gland was revealed by the injection of insulin. The relatively greater amounts of amine in the irradiated rats, taken together with the fewer symptoms of hypoglycaemia produced in them, probably indicates that the irradiated rats were less affected by insulin.

At 68 hours and 72 hours, however, the pressor amines in the irradiated rats were less than in the controls. This might indicate either a disappearance of insulin resistance or a failure of the synthetic activity of the adrenal medulla.

Change in adrenal weight and pressor amines.—The results in the different experiments for adrenal weight and pressor amine content have been grouped according to the time after irradiation at which the irradiated rats were killed. They are shown in Table VIII. The adrenal weights were calculated per 100 g. body weight, and the ratio of irradiated to control figures was calculated for each separate experiment. The same was done for the amounts of pressor amine. Each figure in Table VIII is the mean of all the ratios for the time interval concerned. It is seen

TABLE VIII

ADRENAL WEIGHT AND PRESSOR AMINE CONTENT OF IRRADIATED RATS AS PERCENTAGES OF THE CONTROLS

Interval between irradiation and death (hr.)	48	66–67	70–73	78–80
No. of rats: $\frac{\text{irradiated}}{\text{control}}$	$\frac{2}{2}$	$\frac{4}{3}$	$\frac{9}{7}$	$\frac{4}{4}$
Adrenal wt./100 g. rat as % of control ..	104	133	146	193
Pressor amine/100 g. rat as % af control ..	107	84	62	49

that adrenal hypertrophy in the irradiated rats is not appreciable at 48 hours, but thereafter rises progressively up to 80 hours, when the glands are twice as big as the controls. The same trend is also visible in Table VII. The pressor amine content, expressed in relation to body weight and not in relation to adrenal weight, on the other hand steadily falls to about half the control value in the same time.

Adrenal hypertrophy caused by hexoestrol.—In order to discover whether agents other than irradiation which lead to adrenal hypertrophy also cause the content of pressor amines to diminish, we followed the procedure described by Vogt (1945), implanting tablets of hexoestrol into rats. We used tablets of 250 mg. each, and performed two experiments each on eight rats, four rats receiving a tablet and four being kept as controls. The weights (90–120 g.) and sexes of the rats were similar in the control group and in the group implanted with hexoestrol tablets at the beginning of the experiment. The implanted rats gained much less weight during the experiment ; thus, while the mean control weight rose from 95 g. to 128 g. in one of the two experiments, the mean weight of implanted rats rose from 101 g. to 106 g. All rats were killed 12 days after implantation. The results are given in Table IX. They show that there was no appreciable difference in the mean total amount of pressor amine in the adrenals of the control and of the implanted rats, although the adrenal glands of the implanted rats were 143 per cent of the weight of the controls. This result for the increase of adrenal weight agrees very closely with that of Vogt (1945).

Discussion

Changes in weight and cholesterol content of the adrenal gland of the rat after irradiation have been determined by Patt, Swift, Tyree, and John (1947). The weight changes they recorded after 900 r. were similar to those recorded here after 1,000 r. ; that is to say there was no change after 48 hours, and a substantial increase

TABLE IX

EFFECT OF HEXOESTROL ON ADRENAL WEIGHT AND ON PRESSOR AMINE CONTENT OF ADRENALS

Sex	Control		Implanted	
	Pressor amine per rat (μg.)	Adrenal wt. (mg.)	Pressor amine per rat (μg.)	Adrenal wt. (mg.)
Female	47.5	41.0	42.5	132.0
,, ...	50.0	48.0	62.5	50.0
,, ...	33.2	36.0	36.2	33.0
,, ...	37.8	35.0	34.9	45.0
,, ...	38.5	51.0	33.3	63.0
,, ...	36.0	48.0	30.5	47.0
Male	60.0	30.0	37.5	38.0
,, ...	47.5	27.0	57.5	46.0
Mean ..	43.8	39.5	41.9	57.0

at 72 hours after irradiation. The cholesterol content altered in the same way as the pressor amine content ; at 48 hours both were normal, but by 72 hours both had decreased considerably. Whether there is any relation between cholesterol content and the pressor amine content it is from present knowledge impossible to say.

The increase in adrenal weight and depletion of cholesterol are signs of increased cortical activity. This should also lead to an increased deposition of liver glycogen, as was found by North and Nims (1949) in irradiated rats. Thus increased cortical activity provides one explanation of the increased insulin resistance of irradiated rats, since administration of cortisone or A.C.T.H. produces hyperglycaemia, and an apparent increased resistance to insulin (Conn, Louis, and Johnston, 1949). Liver glycogen is increased as early as 24 hours after irradiation (North and Nims, 1949), and the present work shows that insulin resistance, as judged by symptoms and the pressor amine content of the adrenals, is also present before hypertrophy of the adrenals has begun to occur. However, it is also possible that the early increase in liver glycogen is not a consequence of increased cortical activity, and that the increased insulin resistance is simply due to the increased glycogen store, whatever its cause may be. Although the increase in liver glycogen may not occur in adrenalectomized animals, the adrenal glands may not be directly responsible for it.

Patt, Swift, Tyree, and Straube (1948) found that hypophysectomy reduced the changes in adrenal weight and cholesterol content which followed *x*-irradiation of rats. The polyuria and polydipsia reported by Nims (1950) also suggest an alteration in pituitary function caused by whole-body irradiation.

This evidence is most easily interpreted by assuming that whole-body irradiation is merely one form of " stress " and that the changes in the adrenal are those characteristic of non-specific stress and mediated, at least in part, by the pituitary gland. There is as yet, however, no information on the effects of other forms of stress on the pressor amine content of the adrenals, and other evidence on the part played by the adrenals in the syndrome of whole-body irradiation is contradictory. Adrenalectomy has been stated (1) to have no effect (Straube, Patt, Tyree, and Smith, 1949) and (2) to make mice more sensitive (Chapman and Cronkite, 1950). Similarly, treatment by adrenal extracts has been said (1) to reduce mortality

(Ellinger, 1947; Graham, Graham, and Graffeo, 1950), and (2) to have no effect (Straube, Patt, Tyree, and Smith, 1949; Smith, Smith, and Thompson, 1950).

The cause of death after single doses of radiation is still obscure. Anaemia is usually insufficient in degree to be the immediate cause and infection is often not demonstrable. On the evidence given here it is not possible to say whether the deficiency in medullary pressor amines is a real contributory cause of death, or whether the deficiency is merely one consequence among many of a metabolic failure which is followed by death for some other reason.

Summary

1. The content of pressor amines (*nor*adrenaline and adrenaline) of the adrenal gland of rats is progressively reduced after a lethal dose of *x*-irradiation of the whole rat. The reduction begins after 48 hours, and by 78–80 hours the content is about 50 per cent of normal.

2. The dopa-decarboxylase activity of liver extracts from irradiated rats is also reduced to some extent. Normal enzymic activity can, however, be restored *in vitro* by the addition of pyridoxal phosphate to the liver extract.

3. The fall in dopa-decarboxylase in the liver after irradiation is lessened by adding pyridoxine to the diet, but the fall in pressor amine is unchanged.

4. When the irradiated rats are injected with insulin from 48 hours onwards, they show fewer symptoms of hypoglycaemia than the controls, and their adrenal glands are less depleted of pressor amine as a result of the action of insulin than are those of the controls. This is true for the period 48 to 64 hours after irradiation. At 68 and 72 hours after irradiation the position is reversed, and the glands of the irradiated rats are more depleted by insulin than the glands of the controls.

5. After irradiation there is a progressive hypertrophy of adrenal glands beginning about 48 hours. This hypertrophy progresses as the pressor amine content declines.

6. When tablets of hexoestrol are implanted into rats, the adrenal glands hypertrophy as was shown by Vogt. This hypertrophy is not accompanied by any change in the pressor amine content.

References

Blaschko, H. (1939). *J. Physiol.*, **96**, 13*P*.
Blaschko, H., Carter, C. W., O'Brien, J. R. P., and Sloane Stanley, G. H. (1948). *J. Physiol.*, **107**, 18*P*.
Burn, J. H., Finney, D. J., and Goodwin, L. G. (1950). *Biological Standardization*, 2nd ed. London: Oxford University Press.
Burn, J. H., Hutcheon, D., and Parker, R. H. O. (1950). *Brit. J. Pharmacol.*, **5**, 417.
Conn, J. W., Louis, L. H., and Johnston, M. W. (1949). *J. Lab. clin. Med.*, **34**, 255.
Chapman, W. H., and Cronkite, E. P. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 337.
Ellinger, F. (1947). *Proc. Soc. exp. Biol., N.Y.*, **64**, 31.
Goldfeder, A., Cohen, L., Miller, C., and Singer, M. (1948). *Proc. Soc. exp. Biol., N.Y.*, **67**, 272.
Graham, J. B., Graham, R. M., and Graffeo, A. J. (1950). *Endocrinology*, **46**, 434.
Holtz, P., Heise, R., and Lüdtke, K. (1938). *Arch. exp. Path. Pharmak.*, **191**, 87.
Manfield, J. R., McIlwain, A. J., and Robertson, J. E. (1943). *Radiology*, **41**, 383.
Nims, L. F. (1950). Oral communication, Cinquantenaire de la Découverte de Radium, Paris.
North, N., and Nims, L. F. (1949). *Fed. Proc.*, **8**, 119.
Patt, H. M., Swift, M. N., Tyree, E. B., and John, E. S. (1947). *Amer. J. Physiol.*, **150**, 480.
Patt, H. M., Swift, M. N., Tyree, E. B., and Straube, R. L. (1948). *Science*, **108**, 475.
Shorvon, L. M. (1949). *Brit. J. Radiol.*, **22**, 49.
Sloane Stanley, G. H. (1949). *Biochem. J.*, **45**, 556.
Smith, W. W., Smith, F., and Thompson, E. G. (1950). *Proc. Soc. exp. Biol., N.Y.*, **73**, 529.
Straube, R. L., Patt, H. M., Tyree, E. B., and Smith, D. E. (1949). *Proc. Soc. exp. Biol., N.Y.*, **71**, 539.
Vogt, M. (1945). *J. Physiol.*, **104**, 60.

ARCHIVES OF DISEASE IN CHILDHOOD
BRITISH JOURNAL OF VENEREAL DISEASES
BRITISH JOURNAL OF INDUSTRIAL MEDICINE
ANNALS OF THE RHEUMATIC DISEASES
JOURNAL OF NEUROLOGY NEUROSURGERY AND PSYCHIATRY
THORAX
BRITISH JOURNAL OF SOCIAL MEDICINE
BRITISH JOURNAL OF PHARMACOLOGY AND CHEMOTHERAPY
JOURNAL OF CLINICAL PATHOLOGY
ABSTRACTS OF WORLD MEDICINE
A Monthly Critical Survey of Periodicals in Medicine and its Allied Sciences
BRITISH HEART JOURNAL
ABSTRACTS OF WORLD SURGERY OBSTETRICS & GYNAECOLOGY
A Monthly Critical Survey of Periodicals in Medicine and its Allied Sciences

BRITISH MEDICAL JOURNAL

Every Saturday. Sub. £4 4s. 0d. a year

ABSTRACTS OF WORLD MEDICINE

Monthly, £3 3s. 0d. p.a Single Copy 6/-

ABSTRACTS OF WORLD SURGERY OBSTETRICS & GYNAECOLOGY

Monthly, £2 2s. 0d. p.a Single Copy 4/-

THE BRITISH JOURNAL OF OPHTHALMOLOGY

Monthly, £4 4s. 0d. p.a.

If taken with Ophthalmic Literature (a comprehensive abstract of ophthalmology and cognate literature)—£6 6s. 0d. a year

The undermentioned are published Quarterly at £1 10s. 0d. a year each

BRITISH JOURNAL OF PHARMACOLOGY AND CHEMOTHERAPY
BRITISH JOURNAL OF INDUSTRIAL MEDICINE
BRITISH HEART JOURNAL
BRITISH JOURNAL OF VENEREAL DISEASES
THORAX
JOURNAL OF NEUROLOGY, NEUROSURGERY AND PSYCHIATRY
BRITISH JOURNAL OF SOCIAL MEDICINE
ANNALS OF THE RHEUMATIC DISEASES
JOURNAL OF CLINICAL PATHOLOGY

MEDICAL AND BIOLOGICAL ILLUSTRATION

Published Quarterly at £2 2s. 0d. a year

ARCHIVES OF DISEASE IN CHILDHOOD

Published six times a year at £2 2s. 0d. a year

Subscriptions to: Publishing Dept. B.M.A. House, Tavistock Sq. London, W.C.1

CONTENTS

PAGE

The Renal Effects of Urethane and Colchicine in Adult Rats S. E. DICKER 169

An Improved Apparatus for the Study of the Motility of Isolated Organs CAMILLO BIANCHI and GIOVANNI CERIOTTI with the technical assistance of MARIO ORTELLI 182

2 : 4-Diaminopyrimidines—A New Series of Antimalarials E. A. FALCO, L. G. GOODWIN, G. H. HITCHINGS, I. M. ROLLO, and P. B. RUSSELL 185

Cytotoxic Agents: I, Methylolamides with Tumour Inhibitory Activity and Related Inactive Compounds
J. A. HENDRY, F. L. ROSE, and A. L. WALPOLE 201

Cytotoxic Agents: II, Bis-Epoxides and Related Compounds
J. A. HENDRY, R. F. HOMER, F. L. ROSE, and A. L. WALPOLE 235

Inhibition of Amine Oxidase by *Iso*thiourea Derivatives
F. N. FASTIER and JOYCE HAWKINS 256

The Mode of Action of Morphine upon the Intestine
E. M. VAUGHAN WILLIAMS and D. H. P. STREETEN 263

The Duration of Cocaine Anaesthesia in the Rabbit Cornea. A Statistical Examination PETER A. YOUNG 273

*Nor*adrenaline Output in Urine After Infusion in Man
U. S. VON EULER and R. LUFT 286

Insulin and the Suprarenal Gland of the Rabbit G. B. WEST 289

The Effect of Adrenaline, *Nor*adrenaline, and *Iso*propyl *Nor*adrenaline on the Ascorbic Acid Content of the Rat's Adrenal I. G. JARRETT 294

The Pharmacology of the Toxiferines W. D. M. PATON and W. L. M. PERRY 299

Reversal of Nicotine Action on the Intestine by Atropine
N. AMBACHE and J. EDWARDS 311

Enzymes and their Substrates in the Adrenal Gland of the Ox H. LANGEMANN 318

The Mode of Action of Antrycide W. E. ORMEROD 325

A Study of Basophilic Inclusion Bodies Produced by Chemotherapeutic Agents in Trypanosomes W. E. ORMEROD 334

The Pressor Amines in the Adrenal Medulla after Irradiation
C. T. ANDERSON, H. BLASCHKO, J. H. BURN, and R. H. MOLE 342

PRINTED IN GREAT BRITAIN BY FISHER, KNIGHT AND CO., LTD. THE GAINSBOROUGH PRESS, ST. ALBANS.